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REMARKS

Claims 35-41, 44 and 60-68 are herein amended. Attached hereto is a marked-up version of the changes made by the current amendments, captioned "Version With Markings To Show Changes Made." The amendments are fully supported by the specification and claims as originally filed. Thus, no new matter has been added.

Claims 35-54 and 60-70 are currently pending. Applicants respectfully request reconsideration of the pending rejections in view of the amendments and remarks made herein.

I. Objection to the Specification

The Examiner has objected to the specification because the description of Figure 3 and the content of Figure 3 do not match. Applicants sincerely thank the Examiner for pointing out this clerical error. In response, Applicants have amended the specification to correct this obvious clerical error, and to point out that Figure 3 depicts the purification of the protein of the invention on a gel. Applicants submit that the content of Figure 3 would have been obvious to one of skill in the art upon reviewing the figure. Accordingly, no new matter has been added.

II. Rejections Under 35 U.S.C. § 112, First Paragraph for Enablement

The Examiner has rejected claims 35-54 and 60-70 under 35 U.S.C. § 112, first paragraph as allegedly not being enabled by the specification. Specifically, the Examiner contends

> Miranda et al. disclose only attraction and not activation neutrophils and macrophages, and this feature of S100A12 has not been indicated to be generic to include leukocytes as well. Assuming for the sake of argument that the Applicants have

> extrapolated the potential of the instant CCI to attract and activate leukocytes as well, the specification fails to disclose as to how to use the protein to treat acute, or resistant chronic infections.

and

> the disclosure fails to provide guidance of how to use the variant polypeptides generated while citing general features, without specific details such as (i) what are the 30 or 50 contiguous amino acids to select from in generating polypeptides as recited in claims 35-41, or what are the permitted amino acid changes to obtain a first amino acid with 90% or more identical to a second amino acid sequence selected from the either the polypeptide of SEQ ID NO:2 or from a polypeptide encoded by the cDNA of the ATCC Deposit No. 97304.

See Paper No. 15, page 4.

Applicants respectfully disagree and traverse, and herein address each of the above points in turn.

Preliminarily, Applicants point out that exact methods for using the claimed compound to treat a specific asserted disease, *i.e.* acute or resistant chronic infections, are not necessary to satisfy the requirements of 35 U.S.C. § 112, first paragraph. Indeed, the Federal Circuit has held that knowledge of, *inter alia*, dosage levels, patient populations, or administration routes is not required to enable claims directed to a compound with pharmacological activity, rather than to a method of treatment using such a compound. *See, e.g., Cross v. Iizuka*, 753 F.2d 1040, 224 U.S.P.Q. 739 (Fed. Cir. 1985). The Court also held that one skilled in the art could determine such details without undue experimentation, even in 1978. Applicants respectfully note that the level of skill in the art has increased greatly since 1978, making the experimental determination of such details even more routine. Further, the PTO acknowledges that:

> If a statement of utility in the specification contains within it a connotation of how to use, and/or the art recognizes that standard

> modes of administration are known and contemplated, 35 U.S.C. 112 is satisfied. ... For example, it is not necessary to specify the dosage or method of use if it is known to one skilled in the art that such information could be obtained without undue experimentation. If one skilled in the art, based on knowledge of compounds having similar physiological or biological activity, would be able to discern an appropriate dosage or method of use without undue experimentation, this would be sufficient to satisfy 35 U.S.C. 112, first paragraph.
>
> M.P.E.P. § 2164.01(c).

The instant situation is directly analogous to that of *Cross v. Iizuka* for two reasons. First, the instant claims are drawn to a compound (CCI) that possesses a proven pharmacological activity (leukocyte attraction), rather than to a method of treatment. Thus, information such as dosage and administration routes is not required to enable the use of the claimed compound. Second, even if the instant claims were directed to methods of treatment, the determination of details such as dosage level and administration routes is well within the scope of the skilled artisan. *See, e.g., Cross v. Iizuka.* Accordingly, the instant rejection cannot be maintained.

Moreover, Applicants submit that methods for using polypeptides that mediate the recruitment of leukocytes were well known in the art at the time the instant application was filed and, as such, the skilled artisan would clearly be enabled to practice at least one use of the claimed polypeptides without undue experimentation.

For example, Luo et al. (Journal of Immunology, 153:4616-4624 (1994), previously submitted as reference C12 with the Information Disclosure Statement filed February 8, 2000) describe an assay for the isolation of peritoneal and peripheral blood leukocytes using TCA3, a chemokine known to mediate the recruitment of leukocytes (*see* p.4617). In this assay, intraperitoneal injection of purified recombinant TCA3 causes a rapid influx of leukocytes to the peritoneal cavity within as little as two hours. Leukocytes are then routinely isolated from peritoneal

exudates, washed, and resuspended in basic media. This type of assay has tremendous value for *in vitro* studies on the effects of leukocyte aggregation on disease.

Additionally, chemokines have routinely been used as a standard for identifying novel leukocyte chemotactic agents. For example, Hara et al. (Journal of Immunology, 155:5352-5358 (1995); previously submitted as reference C8 with the Information Disclosure Statement filed February 8, 2000) used a known mediator of leukocyte recruitment, MIP-1α, as a positive control in determining whether a novel compound was a leukocyte chemoattractant.

Thus, it is clear that at the time the present application was originally filed, routine, art-recognized techniques were available for using known mediators of leukocyte recruitment. Accordingly, Applicants submit that the skilled protein chemist or molecular biologist, enlightened by the teachings of the present specification, was more than capable of using the CCI polypeptides of the invention in at least one, if not all, of the aforementioned assays, as well as in other assays known in the art, and for the uses described in the specification. Accordingly, the enablement requirement has been satisfied.

With regard to the Examiner's second assertion, *i.e.*, that "it would be undue experimentation for one of skill in the art to make these undescribed variants of SEQ ID No.2 that would be functionally equivalent to what is expected of the instant CCI," Applicants respectfully note that § 112, first paragraph only requires Applicants to enable the skilled artisan to make and use that which is claimed. Applicants disagree with the Examiner's implication that a limitation is present in the claims requiring that the claimed polypeptides be 'functionally equivalent' to the full-length CCI

polypeptide. (*See*, Paper No. 15, page 5). Applicants point out that nothing in the instant claims requires that the claimed polypeptides retain such activity, and the Examiner has not provided any support for such an interpretation of the instant claims. It is improper to read a limitation into a claim from the specification. *See, e.g.*, M.P.E.P. § 2111 at 2100-36 to 37; *In re Van Geuns*, 988 F.2d 1181, 26 U.S.P.Q.2d 1057 (Fed. Cir. 1993).

Applicants emphasize that the specification does enable the use of the claimed polypeptides that retain the biological activity of the full-length CCI polypeptide. However, Applicants further emphasize that the instant specification also describes and teaches uses of the claimed polypeptides that do not require such activity, for example, as an immunogen to produce antibodies. *See* page 35, last paragraph. Thus, since the instant claims do not contain any limitation requiring the claimed polypeptides to retain the biological activity of the full-length CCI polypeptide, whether the polypeptides retain such biological activity or not is irrelevant, so long as the specification enables a person of ordinary skill in the art to practice a single use of the claimed polypeptides without undue experimentation. *See, e.g.*, M.P.E.P. § 2164.01(c).

Undue experimentation is experimentation that would require a level of ingenuity beyond what is expected from one of ordinary skill in the field. *Fields v. Conover*, 170 USPQ 276, 279 (C.C.P.A. 1971). The factors that can be considered in determining whether an amount of experimentation is undue have been listed in *In re Wands*, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). Among these factors are: the amount of effort involved, the guidance provided by the specification, the presence of working examples, the amount of pertinent literature and the level of skill in the art.

The test for undue experimentation is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine. *Id.*

Applicants respectfully submit that the Examiner has not provided sufficient evidence or a basis to question the enablement provided in the specification for the claimed polypeptides. In particular, since the Examiner's analysis and argument depends upon the improper limitation of the instant claims to polypeptides with the biological activity of the full-length CCI polypeptide, the Examiner has not shown why the skilled artisan would not be enabled to practice any use of the claimed invention. Applicants submit that the present disclosure contains a teaching of how to make and use the invention, which must be taken as enabling absent contrary evidence. *See, e.g.,* M.P.E.P. § 2164.05.

Applicants submit that in the instant application, the disclosed or otherwise known methods of making polypeptides may be used to make and then determine, without undue experimentation, whether a given polypeptide encompassed by the claims can be used to, for example, mediate the recruitment of leukocytes, or to generate an antibody which specifically binds to the full-length CCI polypeptide, the enablement requirement is fully satisfied. *See In re Wands*, 8 U.S.P.Q.2d at 1404; *Ex parte Mark*, 12 U.S.P.Q.2d 1904, 1906-1907 (B.P.A.I. 1989).

Moreover, methods for altering a nucleotide sequence to substitute amino acid residues in the encoded amino acid sequence, while maintaining functional qualities of the original protein, were also well known in the art as of the relevant date of December 8, 1995. For example, Ikeda *et al., J. Biol. Chem.* 267: 6291-6296 (1992) (submitted herewith as Exhibit A) describes the use of region-specific mutagenesis to map epitopes of the recA protein. In addition to identifying which alterations can be

tolerated without affecting the antigenicity of a protein, the authors note that this technique facilitates "location of functionally active sites on the tertiary structure of the protein" (Ikeda et al., abstract). The authors conclude in the final paragraph at page 6296:

> The technique of region-specified random base substitution involving the use of PCR employed in this study is very useful not only for epitope mapping, as described in this paper, but is also widely useful for studies of the function of a gene and an enzyme or protein, because of the flexibility as to specifying a target region, and the high yield of the random base substitutions.

The Ikeda et al. method of was modified to be even more expedient, as reported by Stuurman *et al., Journal of Cell Science* 108: 3137-3144 (1995) (submitted herewith as Exhibit B). In mapping the phosphorylated epitope of *Drosophila* lamin, the authors concluded that their random mutagenesis and screening method was "both rapid and efficient" (Sturrman et al., at page 3143, right column). Thus, making variants of a known sequence that retain functionality was entirely *routine* at the time the application was filed.

Regardless, Applicants submit that the specification does indeed provide sufficient guidance to enable the skilled artisan to know which regions of the disclosed polypeptide are responsible for the interactions underlying its biological functions. In addition to disclosing the amino acid sequence, the specification at page 6, line 28, through page 7, line 26, and at Figures 1 and 2, provides a detailed analysis of the structural and functional attributes of CCI, including, for example, regions of similarity with the well characterized, related pig calgranulin C protein. Knowledge of such regions of a polypeptide can serve as a guide to the skilled artisan, identifying those regions that tolerate only relative conservative substitutions or no substitutions.

Thus, one of skill in the art would be able to predict with reasonable certainty which variants would have activity comparable to the one disclosed.

In light of the above remarks, Applicants submit that methods for to make any variant (including substitution, deletion, insertion or any combination thereof) that retains the biological activity of SEQ ID NO:2 were well known in the art at the time the instant application was filed, and that the experimentation necessary for doing so is entirely routine for the skilled artisan. Further, the teachings of the specification would enhance the knowledge of the skilled artisan by providing guidance as to which regions of the protein would be tolerant of modification and which were not. Finally, in regards to § 112, first paragraph, the M.P.E.P. states that the "evidence provided by applicant need not be conclusive but merely convincing to one skilled in the art." M.P.E.P. § 2164.05. Applicants put forward that for the reasons set forth above, one skilled in the art would be convinced by this evidence.

Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw the rejection of claims 33-54 and 60-70 under 35 U.S.C. § 112, first paragraph.

III. Rejections Under 35 U.S.C. § 112, First Paragraph for Written Description

The Examiner has maintained the rejection of claims 42 and 60(c) under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors, at the time the application was filed, had possession of the claimed invention. *See* Paper No. 15, page 5. In particular, the Examiner has rejected claims 42 and 60(c), contending that "... the claims are directed to 'contemplated or

wish to know' aspect of the mature polypeptide of instant SEQ ID NO:2." *Id.* at page 6.

Applicants respectfully disagree and traverse this rejection. In particular, this rejection should be withdrawn because (1) the specification and claims 42 and 60(c) relate to the mature forms of the CCI polypeptide encoded by the deposited clone, as commonly defined by the structure of its precursor, *i.e.*, "the precursor amino acid sequences," which are processed to mature forms of CCI that do not include amino acid residues such as a leader or secretory sequence of the precursor form; and (2) one skilled in the art reading the instant specification would recognize that the inventors had possession of the mature forms of CCI encoded by the deposited clone as claimed.

A. The Specification Describes Mature Forms of CCI

The specification contains a description of the mature forms of CCI that would allow one skilled in the art to visualize or recognize the subject matter of the claimed invention, as required by the relevant caselaw. *See, e.g., Enzo Biochem, Inc. v. Gen-Probe Inc.*, 285 F.3d 1013, 1018, 62 U.S.P.Q.2d 1289 (Fed. Cir. 2002) (citing *University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 1568, 43 U.S.P.Q.2d 1398, 1406 (Fed. Cir. 1997)).

In particular, the specification describes mature forms of CCI as those forms of CCI which are processed and secreted as a result of expression of the defined CCI precursor sequences described by structure (*i.e.*, "the precursor amino acid sequences"). The instant specification describes the mature forms of CCI as those portions of the preprotein (*i.e.*,"the precursor amino acid sequences") having the leader sequence cleaved by the host cell to form the mature forms of the polypeptide.

See, e.g., page 10, lines 9-27. The mature forms of CCI protein are described as those portions of the proprotein that remain following cellular processing of the prosequence. *See id.* The mature forms of the CCI protein to which the claims are directed are thus expressed and proteolytically processed forms of "the precursor amino acid sequences" such that the resulting mature forms of the polypeptide do not include amino acid residues such as the leader or secretory sequence of the proprotein. *See id.* In particular, the specification states that:

> A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.
>
> *Id.* at page 10, lines 20-23.

The Examiner appears to contend that the specific molecular structure is required in order to fulfill the written description requirement, because one skilled in the art would allegedly not be able to envision the exact amino acid sequence of the claimed polypeptides. Applicants respectfully submit that the instant specification describes the molecular structure of the precursor form of CCI (including providing SEQ ID NO:2), and the production of mature forms of CCI in various mammalian expression host cell systems. *See, e.g.*, page 18, lines 14-23 and Example 4 (describing expression in human fibroblasts). Furthermore, the specification characterizes the structure of the mature form of CCI so produced; *i.e.*, the specification expressly states that the mature form of CCI secreted from a host cell lacks the leader or secretory sequence. *See, e.g.*, page 10, lines 15-18. Thus, given the description of the mature forms of "the precursor amino acid sequences", one skilled in the art would be able to envision the mature forms of CCI encompassed by the claims.

B. The Specification Conveys to One Skilled in the Art that Applicants Were in Possession of the Claimed Invention

One of ordinary skill in the art would not only be able to visualize or recognize the identity of the subject matter of the claimed invention, but would recognize that Applicants were in possession of the claimed invention. Applicants respectfully submit that the response to the previously filed Office Action, dated November 15, 2001, demonstrates that based on the description in the instant specification, one of ordinary skill in the art would recognize that the inventors were in possession of the mature forms of CCI encoded by the deposited clone, as required by the relevant caselaw. *See, e.g., Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563, 19 U.S.P.Q.2d 1111, 1117 (Fed. Cir. 1991).

The Examiner contends that since the structure of the mature form may vary depending on the host cell, it is not clear that the applicant was in possession of the "mature form." Applicants respectfully reassert that contrary to the Examiner's opinion, the mature, processed form is an inherent property of the cellular expression of the precursor form of CCI described in the specification. The host cell proteolytic processing of the mature form is an inherent property conferred by the structure of the precursor form of CCI – the capacity of the precursor to be processed is a consequence of its native sequence (also described in the specification as SEQ ID NO:2), even if the processing may vary depending on the expression system used. Thus, the capacity of the CCI polypeptide to be expressed and proteolytically processed to the mature form of the protein is a natural and intrinsic property of that molecule.

Further, Applicants respectfully submit that courts have consistently held that in order to meet the written description requirement, applicants need not utilize any particular form of disclosure to describe subject matter claimed, but only that the description must clearly allow persons of ordinary skill in the art to recognize that the inventor was in possession of what is claimed. *See, e.g., In re Alton,* 76 F.3d 1168, 1172, 37 U.S.P.Q.2d 1578, 1581 (Fed. Cir. 1996). If a person of ordinary skill in the art would have understood the inventor to have been in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate written description requirement is met. *Id.* at 1175, 37 U.S.P.Q.2d at 1583 to 1584. An adequate written description of a claimed invention "requires a precise definition, such as by structure, formula, chemical name, or physical properties, not a mere wish or plan for obtaining the claimed chemical invention." *Eli Lilly*, 119 F.3d at 1566, 43 U.S.P.Q.2d at 1404 (quoting *Fiers v. Revel*, 984 F.2d 1164, 1171, 25 U.S.P.Q.2d 1601, 1606 (Fed. Cir. 1993)). The disclosure must allow one skilled in the art to visualize or recognize the identity of the subject matter of the claimed invention. *See Enzo Biochem*, 285 F.3d 1013 at 1018, 62 U.S.P.Q.2d at 1292 (citing *Eli Lilly*, 119 F.3d at 1568, 43 U.S.P.Q.2d at 1406).

In the instant case, one skilled in the art would recognize that the mature form of CCI was indeed in the inventor's possession. Although the specification does not explicitly define the beginning and end of the processed, mature form of the CCI polypeptide encoded by the deposited clone by amino acid sequence, the common defining features of the genus of the mature form of CCI is adequately described on the basis of its structure, chemical and physical properties, as well as a common

method of production to allow one skilled in the art to visualize and recognize the subject matter of the claimed invention. Moreover, the specification provides the proprotein and methods for expressing the mature form.

C. The Caselaw Cited by the Examiner is Distinguishable from the Facts of the Present Case

In addition, Applicants also take this opportunity to address and explain why the often-cited *Eli Lilly* and *Amgen v. Chugai* cases (as well as the recently decided *Enzo Biochem* case), are of very limited precedential value to the present situation because the facts of each case can be distinguished and are not applicable to the instant factual circumstances.

Eli Lilly involved generic claims to mammalian cDNAs encoding insulin, while the specification disclosed only a single species – the nucleotide sequence for the rat insulin gene. The Federal Circuit found the generic claims lacking in written description because the specification failed to describe structural features commonly possessed by members of the genus. In other words, the disclosure of the rat cDNA sequence alone did not provide enough information to claim the other unknown mammalian sequences. Thus, the court found that the written description requirement was not satisfied for generic claims directed to all mammalian insulin cDNAs, which were unknown, when only one species is disclosed in the specification. *Id.* at 1565, 43 U.S.P.Q.2d at 1403.

Similarly, *Enzo Biochem* involved claims to nucleic acid probes that hybridize to a particular bacterial genome. Not only did the specification fail to describe the probes by sequence, the specification also failed to disclose the sequence of the bacterial genome. Thus, even further removed from *Eli Lilly, Enzo Biochem* was

trying to claim an unknown sequence (*e.g.*, a probe) that was related to another undisclosed sequence (the bacterial genome). The Federal Circuit found the claimed invention lacking in written description. Citing *Eli Lilly*, the Federal Circuit concluded that an adequate written description of genetic material "requires a precise definition, such as by structure, formula, chemical name, or physical properties."

In *Amgen*, Chugai contended that Fritsch was a prior inventor of claims to the EPO gene because he conceived of a generalized approach for screening a DNA library that might be used to identify and clone the human EPO gene of then unknown constitution. The court found that such a conception of an approach that might result in cloning the gene was speculative and clearly not conception of a purified and isolated DNA sequence encoding the gene. The court held that in order for the conception of a process to satisfy the conception of an unknown composition of matter, such as a gene, the process had to be sufficiently specific that one skilled in the relevant art would succeed in cloning the gene. Indeed, expert testimony provided that success in cloning the human EPO gene using the approach set forth in the specification was not assured until the gene was in fact isolated and its sequence known. Thus, the court held that conception of a general process of cloning did not provide conception of an unknown gene. Instead, conception of a gene requires that the inventor have "a mental picture of the structure of the chemical *or is able to define it by its method of preparation, its physical or chemical properties, or whatever characteristics sufficiently distinguish it.*" *Amgen*, 927 F2d at 1206, 18 U.S.P.Q.2d at 1021 (emphasis added).

It is important to recognize that in *Amgen,* Fritsch knew neither the structure nor physical characteristics of the claimed EPO gene and furthermore had only a

speculative method for obtaining the claimed subject matter. The court's holding that adequate conception of the DNA sequence was contingent upon reduction to practice was based on the uncertainties of the method of obtaining the unknown sequence. In contrast, Applicants submit that the instant claimed subject matter has been characterized and defined not only by a method of preparation, but by physical properties (*e.g.* amino acid sequence of the precursor) and biological activity as set forth *supra*. Further, Applicants submit that as described by the instant specification, the processing of the defined precursor to its mature form is neither a speculative nor prophetic method of obtaining the instant claimed subject matter.

Unlike *Amgen, Eli Lilly* or *Enzo Biochem*, the instant claimed subject matter is not unknown. Instead, in the present case the claimed subject matter is defined by the structure of its precursor, (i.e., by its amino acid and nucleic acid sequence) which is processed to biologically active mature forms that do not contain the leader or secretory sequence of the precursor form. Such disclosure would allow one skilled in the art to visualize or recognize the identity of the subject matter of the claim, as discussed *supra.*

Moreover, Applicants invite the Examiners' attention to *In re Edwards*, 568 F.2d 1349, 196 U.S.P.Q. 465 (CCPA 1978), where the court specifically held under factual circumstances similar to the instant circumstances, that describing the claimed invention as a process of making it is sufficient to meet the written description requirement for a compound claim. In particular, the court in *Edwards* held that the primary concern is whether the description requirement has been complied with, not the mode selected for compliance. In *Edwards* the claimed compound was the predominant product of two specifically defined reactions. The description of the two

reactions which resulted in the claimed compound were found to be an adequate written description of the compound because one skilled in the art would be reasonably led to the described reactions and thus, to the claimed compound.

As in *Edwards*, courts have recognized that the primary function of the written description requirement is to insure that an inventor had possession of the claimed subject matter and to allow one skilled in the art to recognize what is claimed. *See In re Blaser*, 556 F.2d 534, 194 U.S.P.Q. 122 (CCPA 1977), *Enzo Biochem*, 285 F.3d 1013, 62 U.S.P.Q. 2d 1289. The written description requirement is satisfied by the disclosure of the claimed subject matter in such a descriptive means, *e.g.*, words, structures, figures and diagrams, to allow one skilled in the art to visualize or recognize the claimed subject matter. *Enzo Biochem*, 285 F.3d 1013.

The Federal Circuit has also noted that the priority application need not use the identical words to describe the claimed invention, if it shows the subject matter claimed with an adequate direction as to how to obtain it. *See Kennecott Corp. v. Kyocera Int'l, Inc.,* 835 F.2d 1419, 1422, 5 U.S.P.Q.2d 1194, 1197 (Fed. Cir. 1987), *cert denied*, 486 U.S. 1008 (1988). It is equally well established that satisfaction of the written description requirement does not require *in haec verba* antecedence in the originally filed application. *See In re Lukach*, 440 F.2d 1263, 169 U.S.P.Q. 795 (CCPA 1971). The written description requirement can be satisfied by showing that the disclosed subject matter, when given its "necessary and only reasonable construction," inherently (*i.e.*, necessarily) satisfies the limitation in question. *Staehelin v. Secher,* 24 U.S.P.Q.2d, 1513, 1520 (Bd. Pat. Int'f. 1992) ("a specification need not describe the exact details for preparing every species within the genus described"). In general, precedent establishes that although the applicant "does not

have to describe exactly the subject matter claimed, the description must clearly allow persons of skill in the art to recognize that [the applicant] invented what is claimed." *In re Gosteli,* 872 F.2d 1008,1012, 10 U.S.P.Q.2d 1614, 1618 (Fed. Cir. 1989).

Given the instant factual circumstances, there can be no doubt that inventors had possession of the claimed invention, as mature forms of a specifically defined precursor are naturally and intrinsically (and not just potentially) generated by their host cells in the manner of the examples provided in the instant specification. The method for obtaining and isolating such mature forms is described in the specification. As evidenced by the description provided in the instant specification, the method provides more than a reasonable expectation that such mature forms can be obtained. The claimed mature forms are obtained as a matter of fact in carrying out the steps of the process disclosed in the specification. Accordingly, an adequate description of a mature form as it is obtained from a host cell is, concomitantly, an adequate description of such claimed mature forms.

In sum, one of ordinary skill in the art would recognize that the Applicants were in possession of the claimed invention, *i.e.*, mature forms of the precursor amino acid sequences of CCI. In view of the teachings of the specification, there can be no question that the claimed invention has been adequately described to allow one skilled in the art to visualize and recognize that which is claimed. Further, it is unnecessary for the specification to explicitly define by amino acid sequence, the beginning and end of the processed, mature form of CCI in order for one skilled in the art to recognize and identify a mature form that is naturally and inherently produced when expressed by a host cell.

Thus, the instant specification contains sufficient information to allow one of ordinary skill in the art to recognize that Applicants have satisfied the written description requirements. Hence, the rejection under 35 U.S.C. §112, first paragraph, should be withdrawn.

IV. Rejection Under 35 U.S.C. § 102(e)

The Examiner has upheld the rejection of claims 35-59 under 35 U.S.C. § 102(e) as being anticipated by Hitomi et al. Specifically, the Examiner contends that the declaration under 37 C.F.R. § 1.131 filed by Applicants is insufficient to overcome Hitomi et al. as a reference because

> The evidence submitted is insufficient to establish a conception of the invention prior to the effective date of the Hitomi et al. reference. While conception is the mental part of the inventive act, it must be capable of proof, such as by demonstrative evidence or by a complete disclosure to another. Conception is more than a vague idea of how to solve a problem. The requisite means themselves and their interaction must also be comprehended. See *Mergenthaler v. Scudder*, 1897 C.D, 724, 81 O.G. 1417 (D.C. Cir. 1897).
>
> In this case, there is not even a vague idea of a problem to be solved. Under 37 CFR 1.131, applicant may show any of the following to antedate a reference:
>
> A) reduction to practice prior to the effective date of the reference;
>
> B) conception prior to the effective date of the reference coupled with subsequent due diligence to reduce to practice, or
>
> C) conception of the invention prior to the effective date of the reference coupled with due diligence from prior to the reference date to the filing date of the application. See M.P.E.P. 715.07.

See Paper No. 15, page 7.

Applicants respectfully disagree and traverse this rejection.

Preliminarily, Applicants respectfully note that the Examiner is applying the improper standard for the instant situation. The requirements cited by the Examiner

are for establishing prior invention of subject matter, such as in interference or infringement proceedings. However, the purpose of filing a declaration under 37 C.F.R. § 1.131 is not to demonstrate prior invention, *per se*, but merely to antedate the effective date of a reference. M.P.E.P. 715.07; *See also In re Moore*, 170 U.S.P.Q. 260 (C.C.P.A. 1971). To antedate the effective date of a reference, an applicant need only show as much of the claimed invention as the reference happens to show. M.P.E.P. § 715.02; *see also In re Stempel*, 113 U.S.P.Q. 77; *In re Stryker*, 168 U.S.P.A. 372 (C.C.P.A. 1971). Stated differently, if a cited reference shows A, it is improper to require an applicant to show A plus B to overcome it. *Id. See also In re Wilkinson*, 134 U.S.P.Q. § 171 (C.C.P.A. 1962); *In re Moore*, 170 U.S.P.Q. 260 (C.C.P.A. 1971).

In fact, under 37 C.F.R. § 1.131 practice, "proof of a utility must be shown only if the reference discloses a utility." M.P.E.P. § 715.07 (citations omitted). Should a utility be disclosed in the reference, an affidavit without explicit evidence of prior possession of said utility may be sufficient to overcome the rejection provided one of ordinary skill in the art would be reasonably satisfied that the subject matter shown to antedate the reference possessed the alleged utility, for example in the case of obvious utilities. *Id. See also In re Blake*, 149 U.S.P.Q. 217 (C.C.P.A. 1966). Thus, contrary to the Examiner's assertion, proof of prior knowledge of utility need only be submitted if the reference also discloses a utility, and then only if the utility would not be obvious.

The pending claims of the instant application are drawn to polypeptides of the disclosed invention, as shown in SEQ ID NO:2 and as encoded by the deposited clone. In the instant Office Action, the Examiner has maintained the rejection of the

pending claims over Hitomi et al. (U.S. Patent No. 5,976,832), which discloses but does not claim the subject matter claimed by Applicants in the instant application. In light of the standards articulated in the M.P.E.P. and case law, all that is required of Applicants to overcome this rejection is evidence of prior possession of those features of the claimed invention also disclosed by Hitomi et al. that would not have been obvious to one of skill in the art.

Hitomi et al. disclose the identification of a novel bovine protein present in bovine amniotic fluid. Based on homology, the inventors assign the protein to the S100 family of calcium-binding proteins and name it 'calcium-binding protein in amniotic fluid-1' (CAAF1). The inventors describe experiments in which the bovine sequence is used to generate antibodies to detect the presence of the bovine sequence in various tissues. Tissues testing positive for binding of the bovine antibodies include fetal calf skin, mucosa, amnion, and thymus, as well as human esophagus and esophageal cancer tissues.

With regard to the human sequence, Hitomi et al. disclose only the isolation and complete nucleotide sequence of a homologous human sequence and the deduced amino acid sequence (See Example 11, SEQ ID NO:12, and SEQ ID NO:20). The Rule 131 affidavit submitted by applicants shows evidence of prior invention of the nucleotide sequence, as affirmed by the Examiner in the instant Office Action: "In the instant case, the declaration under 1.131 shows only a nucleic acid sequence." Paper No. 15, page 8. However, contrary to the Examiner's statements ("There are no facts or evidence to show that the inventors knew what the sequence was, i.e., any protein encoded thereby . . .". Paper No. 15, page 8), the affidavit does indeed show prior

invention and actual possession of the encoded polypeptide. Specifically, item 3, lines 6-9, of the declaration states that:

> Exhibit C, attached hereto, is a redacted printout of a Batch Worksheet which evidences expression of the protein encoded by clone HALTA54 in a baculovirus expression system. The redacted date upon which this Batch Worksheet was generated is prior to March 09, 1995.

Hence, Applicants have provided evidence that the instant nucleotide sequence was known, and that the polypeptide of the invention had been expressed therefrom, all before March 9, 1995. Accordingly, this evidence is sufficient to antedate Hitomi et al.'s mere disclosure of the sequences.

While Hitomi et al. disclose no additional information specific to the invention in question, the specification does generically indicate that any of the proteins disclosed may be useful in "various assay systems and diagnostic agents for detecting the presence of and quantifying the calcium-binding protein" and "as immunogens for making antiserum or antibodies specific to the calcium-binding protein." *See* Hitomi et al, column 10. Even assuming, *inter alia,* that such description amounted to an assertion of specific, substantial, and credible utilities for the human protein, Applicants assert that such utilities are obvious to one of skill in the art. Indeed, a skilled artisan would clearly know that a given protein may be used to generate antibodies that bind it. A skilled artisan would also know that a given protein may be used to detect the presence of the same protein in a sample. Such utilities did not require any inventive effort on the part of the inventors and would clearly be known by a person of skill in the art upon presentation of the protein sequence. Thus, as affirmed by the M.P.E.P. and the case law, it is not necessary for Applicants to provide evidence in an affidavit under Rule 131 of such utilities.

In light of the above remarks, Applicants submit that the Declaration of Jian Ni, Guo-Liang Yu, Pedro Alfonso, Jeffrey Su, and Reiner Gentz Under 37 C.F.R. § 1.131 submitted with the response filed April 2, 2001, is sufficient to antedate Hitomi et al. Indeed, Applicants have adequately shown prior invention of the claimed protein and the nucleotide sequence that encodes it. Moreover, Applicants have shown that, to the extent that Hitomi et al. can be said to assert utilities for Applicants' claimed invention (i.e. the human protein disclosed in Hitomi et al.), such utilities would be obvious to one of skill in the art upon mere presentation of the protein sequence or nucleotide sequence encoding it. As discussed above, the M.P.E.P. and case law make it clear that Applicants' declaration under Rule 131 need not disclose a utility unless the reference also discloses a utility, and then only if the utility would not be obvious. Consequently, Applicants' declaration provides antedating evidence for as much as Hitomi et al. show. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw the rejection of claims 35-59 under 35 U.S.C. § 102(e).

Conclusion

Applicants respectfully request that the above-made amendments and remarks be entered and made of record in the file history of the instant application. Applicants believe that this application is in condition for allowance. If in the opinion of the Examiner, a telephone conference would expedite prosecution, the undersigned can be reached at the telephone number indicated below.

If there are any fees due in connection with the filing of this paper, please charge the fees to Deposit Account No. 08-3425.

Dated: July 3, 2002

Respectfully submitted,

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 9, Issue of March 25, pp. 6291-6296, 1992
Printed in U.S.A.

Epitope Mapping of Anti-recA Protein IgGs by Region Specified Polymerase Chain Reaction Mutagenesis*

(Received for publication, October 14, 1991)

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Monoclonal IgGs were shown to be useful for the specific inhibition of a set of activities of the recA protein, a key protein in homologous genetic recombination. The mapping of the epitopes for these IgGs and site-directed mutagenesis based on the mapping will facilitate location of the functionally active sites on the tertiary structure of the protein, which is being solved by means of physicochemical techniques. We developed a novel technique for region-specified mutagenesis and applied the technique to epitope mapping. Using the polymerase chain reaction in the presence of deoxyinosine triphosphate, we introduced random base substitutions specifically into a region of the *recA* gene defined by a pair of primers. RecA mutants exhibiting altered antigenicity were selected, in plaque-immunoblotting experiments, from libraries of mutagenized *recA* genes constructed on the λgt11 expression vector. Mutant recA genes were obtained at the frequency of about 10^{-2} among the plaques expressing fused recA genes and then each one was expressed as a whole protein, which was characterized by enzyme-linked immunosorbent assay. Analyzing the DNA sequences of the mutant *recA* genes, we located at the amino acid sequence level the epitopes for two anti-recA IgGs which could not be located in previous studies. One of the antibodies was shown to prevent self-assembly of the recA protein and the other was suggested to inhibit the binding of double-stranded DNA. Thus, the active sites involved in these functions would be located in the space around or near the relevant epitope.

The recA protein, and its prokaryotic and virus (T4 phage) analogues promote "homologous pairing" and "strand exchange" between homologous double-stranded and single-stranded DNAs through ATP (or dATP-)-dependent reactions *in vitro*, and were shown to play an essential role in homologous genetic recombination *in vivo*. Homologous pairing is the formation of an intermolecular duplex ("heteroduplex") between a couple of homologous single-stranded and double-stranded DNAs, and strand exchange is the processing of the heteroduplex, such as its elongation. Each of these reactions consist of a number of substeps and the recA protein or its analogues appear to have various active sites that promote each of these substeps, such as an ATP-binding site, ATPase catalytic center, binding site for single-stranded DNA, binding site for double-stranded DNA, and sites for self-polymerization. The localization of these active sites on the tertiary structure of the recA protein is essential for understanding the mechanisms of the underlying biochemical functions of the protein. However, only sites related to ATPase have been partly located at the amino acid sequence level. The mapping of mutation sites as well as x-ray crystallographic analysis of the protein are the main means to this end. A series of our studies involving the use of anti-recA protein monoclonal IgGs is also an approach to the same goal (see Shibata *et al.*, 1991, for review).

We have isolated clones of mouse hybridomas which produce anti-recA protein IgGs (Makino *et al.*, 1985). Two (ARM193 and ARM191) of these anti-recA protein IgGs each inhibit a set of activities of the recA protein without affecting the others; *i.e.* ARM193 severely inhibits the unwinding of the double helix and strand exchange, but allows homologous pairing and single-stranded DNA-dependent ATP hydrolysis (Ikawa *et al.*, 1989; Makino *et al.*, 1985, 1987). On the other hand, ARM191 inhibits the homologous pairing and unwinding of the double helix, but only affects the single-stranded DNA-dependent ATP hydrolysis a little (Makino *et al.*, 1985). ARM193 was suggested to affect the site for the interaction between recA polypeptides (Ikawa *et al.*, 1989) and ARM191 to affect the site on the recA polypeptide for the binding to double-stranded DNA (Makino *et al.*, 1985). We preliminarily located the epitopes for both ARM193 and ARM191 in a C-terminal 88 amino acid region (Phe^{260}-Glu^{347}) of the recA polypeptide by examining the cross-reaction of proteolytic fragments. However, we failed to map them more precisely, since none of the subfragments of the 88-amino acid region exhibited significant cross-reaction with either of the IgGs (Ikeda *et al.*, 1990). Therefore, it was necessary to introduce another technique to overcome the problem. Here, we describe a novel technique for region-specified mutagenesis and, as an application of this technique, the mapping of the epitopes of ARM193 and ARM191 at the amino acid sequence level in distinct but slightly overlapped subregions in the C-terminal 88-amino acid region.

MATERIALS AND METHODS

recA Protein—The purified recA protein was fraction V prepared as described (Shibata *et al.* 1981).

Oligonucleotides—Oligonucleotides were synthesized with a DNA synthesizer (Du Pont-New England Nuclear CODER300) and purified with NENSORB PREP (Du Pont-New England Nuclear).

Techniques for Cloning of DNAs—Treatment with restriction endonucleases and DNA ligase, and the isolation and cloning of the DNA fragments onto vectors were carried out as described (Berger and Kimmel, 1987; Maniatis *et al.*, 1982).

Antibodies and Immunochemical Techniques—The anti-recA pro-

* This research was supported by a grant from the Biodesign Research Program from RIKEN (The Institute of Physical and Chemical Research). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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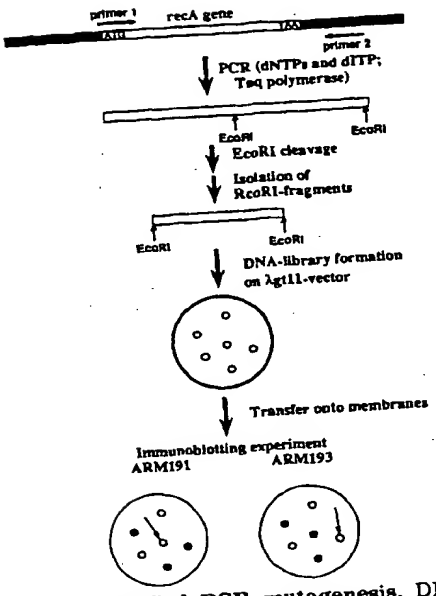

FIG. 1. **Region-specified PCR mutagenesis.** DNA encoding the *E. coli recA* gene flanked by primers 1 and 2 was amplified by PCR in the presence of dITP with the use of *Taq* DNA polymerase. *Eco*RI fragments of the amplified DNA which encoded the C-terminal region of the recA protein were cloned on a λgt11 expression vector to construct DNA libraries of the mutagenized *recA* genes. With appropriate orientation of a fragment relative to the vector, the C-terminal region of the *recA* gene was connected to the *lacZ* gene in-frame. Proteins expressed in the plaques obtained from the libraries were transferred to a pair of membranes and then the cross-reaction with either anti-recA protein IgG ARM191 or ARM193 was tested. The plaques showing cross-reaction with only one of the IgGs (indicated by *arrows*) were picked up and subjected to further cross-reaction tests. The *closed circles* in the *big circles* at the bottom of the figure denote plaques which showed cross-reaction with the indicated IgG.

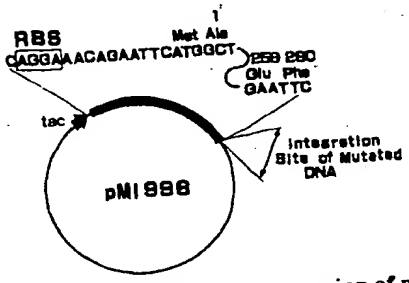

FIG. 2. **Structure of pMI996 for expression of mutated *recA* genes.** DNA encoding the N-terminal region of the recA polypeptide was put under the control of the *Tac* promoter on a multicopy plasmid (pKK223-3). *Eco*RI fragments of the mutagenized *recA* gene were inserted at the *Eco*RI site at the codons for Glu^{259}-Phe^{260}.

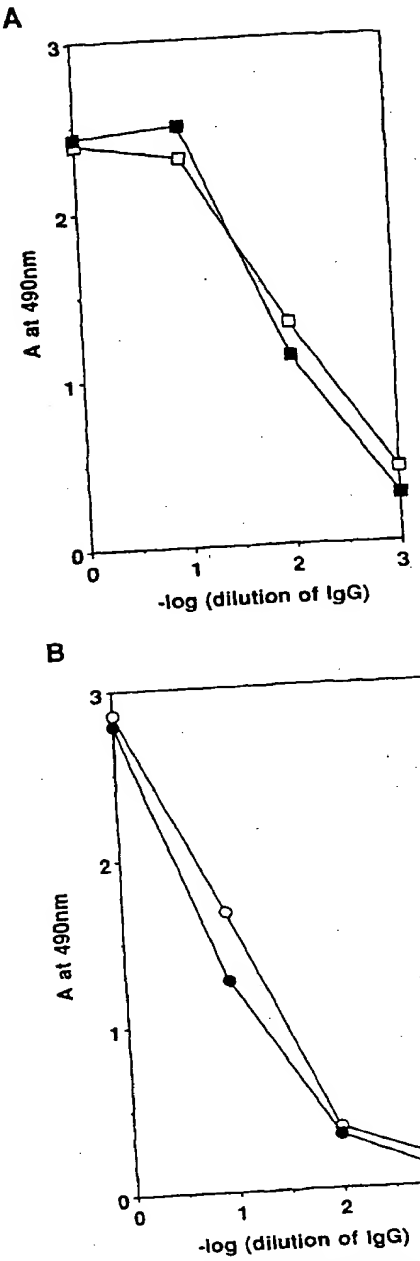

FIG. 3. **Absence of competition in cross-reaction between anti-recA protein IgGs, ARM191 and ARM193.** The wells of a microtiter plate were coated with the purified recA protein. Then, a solution of a tested anti-recA protein (nondilution: 1.5 μg/ml) containing the other IgG was put into the wells and allowed to cross-react with the recA protein. ARM191 belongs to IgG_1 and ARM193 to IgG_{2b}. Thus, the amounts of ARM191 and ARM193 bound to the recA protein were specifically measured by ELISA with the use of an appropriate subclass-specific antibody. *A*: □, the binding of ARM191 in the absence of ARM193; ■, the binding of ARM191 in the presence of 5 μg of ARM193/ml. *B*: ○, the binding of ARM193 in the absence of ARM191; ●, the binding of ARM193 in the presence of 5 μg of ARM191/ml.

tein monoclonal IgGs, ARM191, ARM193, and ARM414, were described previously. We used affinity purified preparations of these IgGs. An anti-recA protein monoclonal IgG, MAb156, was isolated by Karu and Allen (Karu and Allen, 1982), and a purified preparation of this IgG was provided by Dr. Alexander Karu (University of California, Berkeley) and Dr. A. John Clark (University of California, Berkeley).

The enzyme-linked immunosorbent assay (ELISA)[1] was carried out as described previously. Unless otherwise stated, samples of cell-free extracts were diluted in PBS (50 mM potassium phosphate buffer (pH 7.2) containing 150 mM NaCl) and put into the sample wells of a microtiter plate with 96 wells. On the other hand, the concentration of the anti-recA protein IgG was first adjusted to 30 μg of protein/ml ("no dilution") in PBS Tween (PBS containing 0.05% Tween 20) and then a series of dilutions was prepared in PBS/Tween.

[1] The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

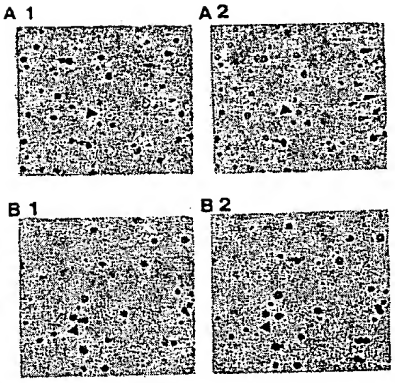

FIG. 4. **Testing of cross-reaction of proteins in plaques obtained from libraries of mutagenized DNA.** The results of two experiments are shown as examples (*A* and *B*). *A1* and *B1* show cross-reaction with ARM191, and *A2* and *B2* that with ARM193. The procedure was described in detail in the legend to Fig. 1. *Arrowheads* indicate mutants which showed altered cross-reaction; mutant D (the top ones in *A1* and *A2*) shows no cross-reaction with ARM191, and mutant 47 (*B1* and *B2*) none with ARM193.

TABLE I

Isolation of mutants by region-specified PCR mutagenesis

dITP	Total plaques expressing recA genes	Number of plaques picked up on the first selection	Total number of mutants identified	Number of species of mutants
μM				
0	1800	5	1	1
0.2	1100	21	4	2
20	2200	5	3	3
200	2000	21	11	10

Testing of the Competition between ARM191 and ARM193—The IgGs, ARM191 and ARM193, belong to subclasses 1 and 2b, respectively (Ikeda *et al.*, 1990), and thus each could be assayed by use of anti-mouse IgG_1 and anti-mouse IgG_{2b} antibodies, respectively. A solution of the purified recA protein (0.2 μg/ml) was put into the wells of a microtiter plate to coat the walls of the wells. Each solution of an indicated amount (no dilution: 1.5 μg/ml) of a tested anti-recA protein IgG (50 μl) contained 0 or 5 μg of the other anti-recA protein IgG/ml. The amounts of the tested anti-recA protein IgG that bound to the wells were measured by ELISA with anti-mouse IgG_1 or anti-mouse IgG_{2b} antibodies (Zymed Laboratories, Inc.).

Region-specified PCR Mutagenesis—An outline of the procedure is given in Fig. 1. pBEU14 DNA, which contains the *Escherichia coli recA* gene (Uhlin and Clark, 1981), was linearized with *Bam*HI and used as the template for PCR (polymerase chain reaction). A DNA region encoding the *recA* gene flanked by primer 1 (5′-ATGGCTATCGACGAAAACAA-3′) and primer 2 (5′-GAATTCTGTCATGGCATATCCTT-3′) was amplified by 25 cycles of PCR (see Fig. 1). Unless otherwise stated, the reaction mixture for PCR comprised 1 μM each of primers 1 and 2, about 3 pM the template DNA, 200 μM each of dATP, dTTP, dGTP, and dCTP, 200 μM deoxyinosine 5′-triphosphate (dITP), 0.025 units of *Taq* DNA polymerase/μl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, and 10 mM Tris-HCl buffer (pH 8.3). Each cycle of PCR consisted of: (i) incubation at 37 °C for 2 min, for loading of the primers onto the template DNA, (ii) incubation at 72 °C for 3 min, for polymerization, and (iii) incubation at 94 °C for 1 min, for denaturation. The amplified DNA was treated with *Eco*RI and the *Eco*RI fragment encoding the C-terminal 93-amino acid region was isolated by gel electrophoresis, followed by trapping on a DEAE membrane (NA45; Schleicher & Schuell). Then, DNA libraries containing the mutagenized *Eco*RI fragments were constructed on the λgt11 expression vector by ligating them at an *Eco*RI site of the vector. With appropriate orientation of a fragment relative to the vector, the C-terminal region of the *recA* gene was connected to the *lacZ* gene in-frame. Plaques of the phages in the libraries were obtained with the *E. coli* Y1090 strain as a host. Proteins in the plaques were transferred to a pair of membranes and then the cross-reaction with either ARM191 or ARM193 anti-recA protein IgG was examined by means of immunoblotting experiments, as described previously (Ikeda *et al.*, 1990; Morishima *et al.*, 1990). The plaques showing cross-reaction with only one of the IgGs (indicated by *arrows* in Fig. 1; examples are shown in Fig. 4) were picked up, and the phages were obtained after repeated single plaque isolation and testing by means of immunoblotting experiments.

DNA Sequence Analysis—The tested DNAs were cloned on pUC119 and then their sequences were analyzed by the dideoxyribonucleotide chain termination method (Sanger *et al.*, 1977); the labeling reaction was carried out by use of the double-stranded template according to a manual for Sequenase (United States Biochemical Co., Cleveland, OH), and the products were analyzed with the use of an automated DNA sequence analyzer (Du Pont GENESIS2000). We analyzed both strands in most of the cases.

Expression of Mutant recA Genes and Preparation of Cell-free Extracts—*Eco*RI fragments which carried mutation(s) in the C-terminal 93-amino acid region were ligated to the *Eco*RI site of the DNA region encoding the N-terminal 260 amino acids, which was under the control of the Tac promoter on a multicopy plasmid, pMI996 (Fig. 2), a derivative of pKK223-3 (Brosius and Holy, 1984). An *E. coli* strain, MV1184 (deletion of the *srl-recA* locus) (Vieira and Messing, 1987), was transformed with the DNA. The transformants were grown at 37 °C to the mid-logarithmic growth phase in 3-ml cultures and then expression of the mutated *recA* genes was induced by isopropyl-β-D-thiogalactoside treatment (0.2 mg/ml) for 2 h. After the treatment, the cells were collected by centrifugation and suspended in 200 μl of a lysis buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 M NaCl, and 5% Triton X-100), and then treated with lysozyme (0.6 mg/ml) at 0 °C for 15 min, followed by the addition of KCl (at 0.24 M). The lysate was centrifuged at about 15,000 × *g* for 15 min and the supernatant of the lysate was saved. The supernatant was diluted about 20-fold with PBS and then subjected to ELISA as described above. In the case of mutants 2 and 38, the precipitates of the lysates were resuspended in 5 M urea containing 100 mM Tris-HCl (pH 7.9), followed by centrifugation, and the mutant proteins were extracted from the precipitates with 100 mM Tris-HCl buffer (pH 7.9) containing 8 M urea and 0.1 M NaCl at 25 °C for 1 h.

RESULTS

Testing of the Competition between ARM191 and ARM193—The preliminary mapping of the epitopes of ARM191 and ARM193 indicated that both epitopes were located between Phe^{260} and Glu^{347} (Ikeda *et al.*, 1990). First, we examined whether or not these anti-recA protein-IgGs showed competition in the cross-reaction with the recA protein. Since ARM191 and ARM193 belong to different IgG subclasses, each can be discriminated through the use of subclass-specific antibodies on ELISA. Fig. 3 indicates that the presence of one of these IgGs did not affect the binding of the other IgG to the recA protein. We conclude that the epitope for ARM191 and ARM193 are different. Thus, we tried to locate the epitopes of these anti-recA protein IgGs more precisely.

Development of Region-specified PCR Mutagenesis and Isolation of Mutants—Since we were not able to locate the epitopes of ARM191 and ARM193 by examinations of the cross-reaction of subfragments of the recA polypeptide, we developed a novel technique for region-specified mutagenesis which could be applied for mapping of the epitopes. The whole process for the isolation of mutants causing altered cross-reaction consists of three stages (Fig. 1); the introduction of region-specified random base substitutions by means of PCR, construction of libraries of the mutagenized DNA with the use of the λgt11 expression vector, and *in situ* testing for cross-reaction of the mutated polypeptides expressed in the plaques obtained from the libraries. In PCR (Saiki *et al.*, 1985), *Taq* DNA polymerase causes the misincorporation of nucleotides (Eckert and Kunkel, 1990). We added deoxyinosine 5′-triphosphate (dITP), at 200 μM, to the reaction mixture for PCR to enhance the misincorporation (Martin and Castro, 1985), and specifically amplified a DNA region defined by a pair of oligonucleotides (primers 1 and 2). Primer 1 includes the initiation codon of the recA polypeptide and

TABLE II

Mutations affecting cross-reaction with anti-recA protein IgG ARM191 or ARM193

Mutation site on recA polypeptide	Name of mutant	Change in DNA sequence	Amino acid replaced	Cross-reactivity on immunoblotting[a]		Half-maximum values on ELISA			
				ARM191	ARM193	ARM191	ARM193	ARM414	MAb156
						μg/ml			
Wild type				+	+				
Experiment 1						0.017	0.27	0.12	0.019
Experiment 2						0.085	0.21	0.19	0.017
Experiment 3						0.021	0.75	0.12	0.012
Average of "plus" recA polypeptide (S.D.)						0.023 (0.024)	0.28 (0.18)	0.14 (0.11)	0.015 (0.004)
283	38	CTG → C**C**G[b]	Leu → Pro	−	+	0.13	0.17	0.075	>6.0
291	D	TAC → T**G**C	Tyr → Cys	−	+	>30	0.27	0.24	>30
296	43	GAG → G**G**G	Glu → Gly	−	+	>30	0.53	0.19	>30
305	4	GCG → G**G**G	Ala → Gly	−	+	0.75	0.30	0.21	1.7
308	32	TGG → TG**C**	Trp → Cys	−	+	>30	0.13	0.21	>30
309	2, 5, 12[c]	CTG → C**C**G	Leu → Pro	−	+	>7.5	0.15	0.053	>30
312	37, 39[c]	AAC → AA**A**	Asn → Lys	−	+	>9.5	0.19	0.11	>9.5
315	30.1[d]	ACC → AT**C**	Thr → Ile	+	−	0.013	>10	0.052	0.015
332	31, 33.1[c,d]	AAC → G**A**C	Asn → Asp	+	−	0.012	>10	0.06	0.013
333	23, 34	TCA → C**C**A	Ser → Pro	+	−	0.015	>10	0.04	0.019
337	47	TTC → T**C**C	Phe → Ser	+	−	NT[e]	NT	NT	NT
338	40	TCT → T**T**T	Ser → Phe	+	−	0.0095	>30	0.075	0.015
294	36.1	AAA → A**G**A	Lys → Arg	+	−	0.013	>10	0.075	0.0075
330	36.2	AAC → G**A**C	Asn → Asp						
319	41.1[d]	ATC → A**C**C	Ile → Thr	−	+	>30	0.085	0.48	>30
323	41.2	GTA → G**C**A	Val → Ala						
267	30.2, 33.2, 36.3[c]	GGT → GG**C**	No replacement						
321	30.3	AAG → AA**A**	No replacement						

[a] Proteins showing the plus phenotype with respect to cross-reaction with the relevant IgG.

[b] Shadow letters indicate bases substituted.

[c] All mutants having the same substitution were isolated from the same PCR preparation, except mutants 23 and 34.

[d] Mutants 30, 33, 36, and 41 have more than two base substitutions, each of which is indicated, *e.g.* 30.1 and 30.2.

[e] NT, not tested.

primer 2 includes a complementary sequence about 80 bases downstream of its stop codon (Fig. 1). Primer 2 was designed so as to have an *Eco*RI cutting site. At more than 200 μM, dITP severely inhibited the amplification of DNA by PCR (data not shown). After 25 cycles of PCR, the amplified DNA was treated with *Eco*RI and the *Eco*RI fragment encoding the region of the recA polypeptide extending from Phe^{260} to the C-terminal Phe was isolated. The fragment was inserted into the *Eco*RI site of the λgt11 expression vector (Young and Davis, 1983). The proteins in the plaques were examined as to their *in situ* cross-reaction with anti-recA protein IgGs ARM191 and ARM193. As shown in Fig. 4, most of the plaques expressing the fused recA polypeptide showed cross-reaction to both IgGs. We could pick up about 1% of these plaques as candidates of mutants which showed cross-reaction to only one of the IgGs (indicated by *arrowheads* in Fig. 4; Table I).

From the selected plaques, phages were recovered and the inserted *Eco*RI fragments were reisolated. The DNA fragments were recloned on the pUC119 vector (Vieira and Messing, 1987) and their DNA sequences were analyzed by the dideoxyribonucleotide chain termination method (Sanger *et al.*, 1977). We analyzed both strands in most of the cases by use of double-stranded form and an appropriate primer. The triplets which were changed by mutations are shown in Table II. During the development of the new technique for mutagenesis, we obtained 19 mutants exhibiting altered cross-reaction to either ARM191 or ARM193 from among the 52 plaques tested (Table I). So far as defined, all mutations were the substitution of amino acid(s) caused by base substitution(s) (Table II). Some of the mutants have a second (third) base substitution which does not affect the amino acid sequence. Of the base substitutions defined, 83% are transitions and 17% transversions.

On the first screening, the addition of dITP appeared to have only a little effect on the PCR mutagenesis (Table I). However, when we characterized the mutants isolated, we found that the addition of dITP is essential for this mutagenesis (Table I). Without dITP or with 0.2 μM dITP, we picked up 5 and 21 plaques, respectively, on the first screening, but obtained only 3 kinds of mutants. When dITP was added at 200 μM, we picked up 21 plaques from among about 2000 plaques expressing the fused recA polypeptide, and from these groups we finally isolated 11 mutants which were categorized into 10 kinds. Three of them (mutants 30, 33, and 36) had base substitutions at two or three sites (see Table II). These findings indicate the significant enhancement of the yield and variation on mutagenesis with dITP, and the very high yield of base substitutions under an appropriate set of conditions.

Some mutants have base substitutions at the same sites. Except mutants 23 and 34 (substitution of Ser^{333}), such overlapped mutants were isolated from the same PCR preparations. Thus, these overlapped mutations seem to be created at an early cycle in PCR and amplified during the procedure.

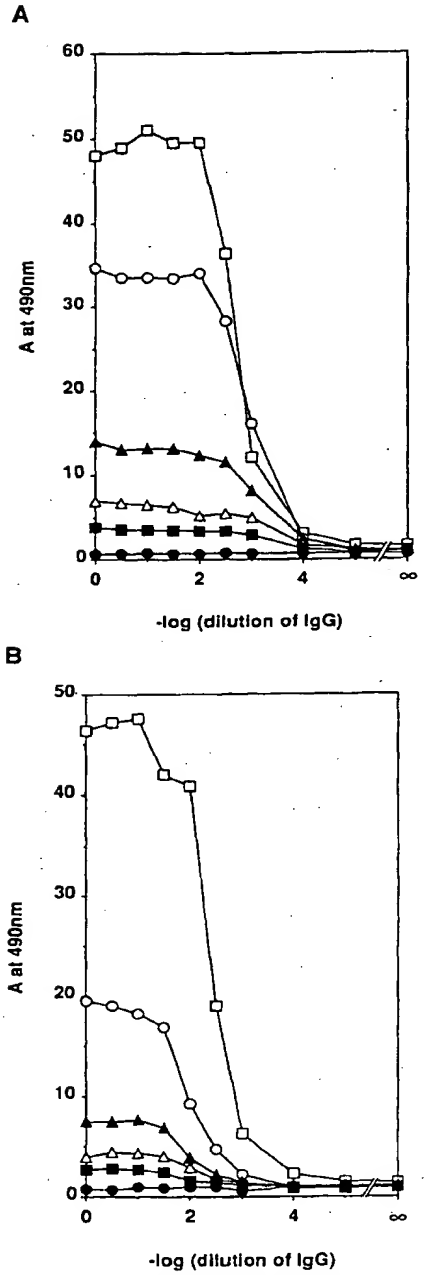

FIG. 5. **Variation of the antigen concentration on ELISA does not change the amount of IgG giving a half-maximum signal.** The wells of a microtiter plate were coated with the purified recA protein at the indicated concentrations. On the other hand, the concentration of the anti-recA protein IgG (ARM191 in *A*; ARM193 in *B*) was adjusted to 30 μg/ml ("nondilution"), and then a series of dilutions of the IgG was put into individual wells. The bound IgG was measured by ELISA. The concentrations of the recA protein were as follows: □, 2 μg/ml; ○, 0.2 μg/ml; ▲, 0.1 μg/ml; △, 0.05 μg/ml; ■, 0.02 μg/ml; ●, without recA protein.

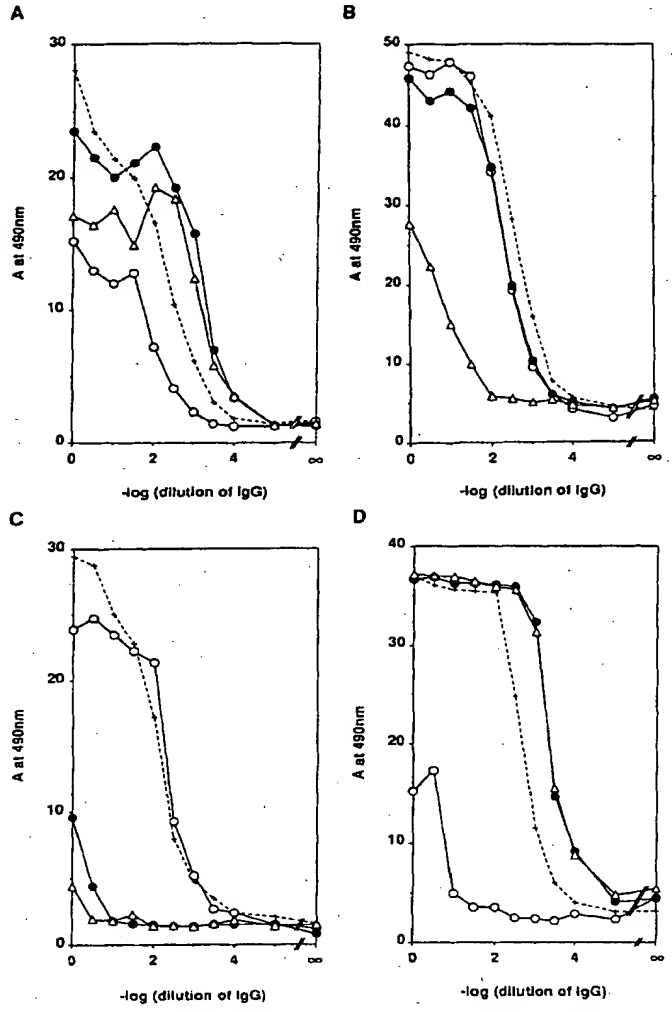

FIG. 6. **Examples of ELISA for testing the cross-reaction of mutant recA proteins to anti-recA protein IgGs, ARM191, ARM193, and MAb156.** Cell-free extracts were prepared from cells in which the mutant *recA* genes were expressed; *A*, wild type; *B*, mutant 38; *C*, mutant 32; *D*, mutant 31. The cell-free extracts were put into individual wells of a microtiter plate to allow the adsorption of proteins. Then, the cross-reaction to each of the IgGs was examined by ELISA, as described in the legend to Fig. 5. The IgGs used were: ●, ARM191; ○, ARM193; △, MAb156. Anti-recA protein IgG, ARM414, of which epitopes is located between Glu^{233} and Lys^{256} (Ikeda *et al.*, 1990) was used as a positive control (+).

Except for in these cases, base substitutions appeared to be introduced at random in the amplified DNA.

Cross-reaction of Mutant recA Proteins—We constructed a plasmid (pMI996; Fig. 2) in which the wild type *recA* gene was under the control of the *Tac* promoter on a multicopy plasmid (a derivative of pKK223-3 (Brosius and Holy, 1984). We replaced the *Eco*RI-*Eco*RI region encoding the C-terminal 93 amino acids of the wild type recA polypeptide with the *Eco*RI fragments on which we found mutation(s) (Fig. 2). Then, a mutant of *E. coli* in which the whole *recA* gene was deleted was transformed with these plasmids. The expression of the mutant *recA* genes was induced and cell-free extracts were prepared, followed by quantitative assaying (ELISA) for cross-reaction with ARM191 and ARM193. For comparison of the extent of cross-reaction of a tested IgG with the recA protein, we determined the amount of the IgG giving a half-maximum signal on ELISA. As shown in Fig. 5, variations in the amount of recA protein did not significantly change the amount of the IgG which gave the half-maximum signal on ELISA. We calculated the amounts of ARM191 and ARM193 giving a half-maximum signal to be 0.0277 (σ = 0.017) and 0.29 (σ = 0.10) μg/ml, respectively, from the data in this figure.

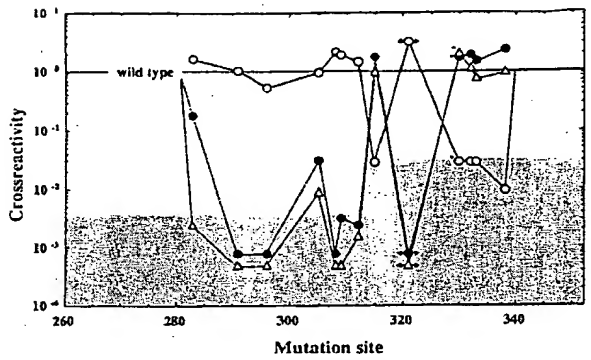

FIG. 7. **Mutation sites and alterations in cross-reactivity.** The *numbers* under the *abscissa* are the amino acid positions from the N terminus of the recA polypeptide. Cross-reactivity is defined as follows: (the amount of an IgG giving the half-maximum value on ELISA with wild-type recA protein)/(the amount of an IgG giving the half-maximum value on ELISA with mutant recA protein). Thus, it shows the deficiency in the cross-reaction. ●, cross-reaction with ARM191; ○, that with ARM193; △, that with MAb156. *Symbols* within the *shadowed area* indicate that no cross-reaction was detected (within the limit of the measurement, which is indicated by each *symbol*). The *symbols* with a *horizontal arrow* around position 320 are those for a mutant having two amino acid substitutions at positions 319 and 323, and those with * at position 330 are those for a mutant having two amino acid substitutions at positions 294 and 330.

Examples of ELISA with mutant recA proteins as well as the wild type protein are shown in Fig. 6. The values calculated on ELISA are listed in Table II. In order to obtain the half-maximum signals on ELISA, all proteins (except mutant 38) which had been determined to be mutants by immunoblotting experiments were shown to require at least 10-fold more IgG ARM191 or ARM193 than the average for plus proteins (Fig. 7). The results shown in Fig. 7 clearly indicate that the mutants affecting the cross-reaction with ARM191 and those affecting that with ARM193 are separately clustered, and only slightly overlap each other (between positions 315 and about 320). This conclusion is consistent with the absence of competition in the binding of these IgGs to the recA protein (Fig. 3).

Comparison of MAb156 with ARM191 and ARM193—MAb156 was isolated by Karu and Allen (Karu and Allen, 1982). Since it was assumed that the epitope of this antibody is located near the C terminus of the recA polypeptide,[2] we examined the cross-reaction of MAb156 with the mutant recA proteins isolated in the present study. All of the mutations affecting the cross-reaction with ARM191 also affected the cross-reaction with MAb156, but none of the mutations affecting the cross-reaction with ARM193 did. This suggests that the epitope for MAb156 and that of ARM191 are similar, but the following results show that they are not identical. Most of the mutations abolishing the cross-reaction with ARM191 also abolished that with MAb156 (Fig. 7), but substitution of Leu^{283} by Pro in mutant 38 strongly interfered with the cross-reaction with MAb156, but not so much with that with ARM191 (Figs. 6*B* and 7). The difference in the mode of cross-reaction between ARM191 and MAb156 was also observed with a mutant recA protein having a substitution of Ala^{305} by Gly (Fig. 7).

DISCUSSION

Region-specific PCR mutagenesis is an efficient tool for introducing random base substitution mutations specifically in a region defined by a pair of primers. From among the plaques expressing the fused *recA* gene, we obtained mutants of the *recA* gene at the frequency of 0.5% under the optimized conditions. Using this mutagenesis, we identified regions of the recA polypeptide in which amino acid substitutions prevent the cross-reaction with ARM191, ARM193, and/or MAb156. The region for ARM191 and that for ARM193 are different, but slightly overlap each other; *i.e.* that for the former IgG comprises positions 283 through about 320, and that for the latter positions through 315–338. Since ARM191 and ARM193 showed no competition, Thr^{315}, and Ile^{319} and Ile^{319} and/or Val^{323} would be located on different sides of a local structure or the whole molecule of the recA polypeptide.

The substitution of an amino acid might interfere with the cross-reaction with an IgG directly or through a very local change in the tertiary structure, or indirectly through extensive alteration of the tertiary structure of the recA protein. The Leu^{283} to Pro substitution in mutant 38 could cause gross alteration of the tertiary structure of the recA protein. The clustering of other amino acid substitutions in a particular region is favorable for the first two possibilities rather than the last one. ARM193 was shown to prevent the self-assembly of the recA protein and ARM191 to inhibit the binding of double-stranded DNA. Thus, the active sites involved in these functions would be located in the space at or around the relevant epitope. We are testing these possibilities by examining the effects of the mutant recA proteins isolated in this study and those constructed by another round of region-specified PCR mutagenesis. Determination of the epitope loci and the inhibitory effects of these IgGs, together with the tertiary structure of the recA protein, will facilitate the understanding of the function of the protein in relation to its structure.

The technique of region-specified random base substitutions involving the use of PCR employed in this study is very useful not only for epitope mapping, as described in this paper, but is also widely useful for studies on the function of a gene and an enzyme or protein, because of the flexibility as to specifying a target region, and the high yield of the random base substitutions.

Acknowledgments—We thank Dr. Alexander Karu (University of California, Berkeley) and Dr. A. John Clark (University of California, Berkeley) for sending us the purified anti-recA protein monoclonal IgG, MAb156, and the related information before publication.

[2] A. J. Clark and A. Karu, personal communication.

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Journal of Cell Science 108, 3137-3144 (1995)
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Interphase phosphorylation of the *Drosophila* nuclear lamin: site-mapping using a monoclonal antibody

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SUMMARY

The *Drosophila* nuclear lamin is highly phosphorylated during interphase. Two interphase isoforms, differing in degree of phosphorylation, can be distinguished by one-dimensional SDS-polyacrylamide gel electrophoresis. One migrates with an apparent mass of 74 kDa (lamin Dm_1); the other is more highly phosphorylated and migrates as a 76 kDa protein (lamin Dm_2). We generated a monoclonal antibody, ADL84 which binds to lamin Dm_1 but not lamin Dm_2. Binding of ADL84 to lamin Dm_2 was restored by phosphatase treatment of immunoblots containing lamins. Immunoprecipitation with ADL84 demonstrated that purified *Drosophila* nuclear lamins Dm_1 and Dm_2 are present as a random mixture of homo- and heterodimers. Indirect immunofluorescence experiments suggest that lamin Dm_1 is present in all *Drosophila* cell types. The epitope for ADL84 was mapped by analyzing binding to bacterially expressed lamin deletion mutants and subsequently by screening for point mutants (randomly generated by polymerase chain reaction) which were not recognized by ADL84. The ADL84-epitope encompasses amino acids R^{22}PPSAGP (arginine 22-proline 28). Peptide competition experiments demonstrated directly that phosphorylation of serine 25 impedes lamin binding by ADL84. This suggests that serine 25 is the lamin Dm_2-specific phosphorylation site.

Key words: lamin, nuclear envelope, phosphorylation

INTRODUCTION

Nuclear lamins are major components of the nuclear lamina, a fibrous layer of proteins underlying the inner nuclear membrane and surrounding the nucleus. In *Xenopus* oocytes, lamins form a network composed of 10 nm fibers which cross-over at regular intervals (Aebi et al., 1986; Whytock et al., 1990). The detailed in situ structure of lamins in other cell-types is not well-known (but see Hill and Whytock, 1993). Lamins interact with the inner nuclear membrane via an isoprenylated carboxy-terminal tail (Weber et al., 1989; Kitten and Nigg, 1991) and/or a lamin-receptor in the nuclear membrane (Worman et al., 1988, 1990; Hennekes and Nigg, 1994). In vitro, lamins also bind chromatin (Burke, 1990; Hoger et al., 1991; Yuan et al., 1991; Glass et al., 1993) and DNA of specific sequence (Ludérus et al., 1992). Results from a number of experiments suggest that lamins play a structural role in nuclear formation (Benavente and Krohne, 1986; Ulitzur et al., 1992) and perhaps in nuclear function (Newport et al., 1990; Meier et al., 1991).

Multiple posttranslational modifications occur on lamins (Nigg, 1992). During mitosis, phosphorylation of one or a few specific lamin sites correlates with lamina disassembly (Smith and Fisher, 1989; Heald and McKeon, 1990; Peter et al., 1990, 1991; Ward and Kirschner, 1990; Goss et al., 1994). Rat liver lamin B was shown to be reversibly demethylated during mitosis (Chelsky et al., 1987). During interphase different stimuli can cause specific lamin phosphorylation via protein kinase C (Hornbeck et al., 1988; Tsuda and Alexander, 1990; Kasahara et al., 1991; Martell et al., 1992; Goss et al., 1994) or other kinases (Molloy and Little, 1992; Eggert et al., 1993). In one case where a biological effect of interphase phosphorylation was demonstrated, Hennekes et al. (1993) showed that phosphorylation of chicken lamin B_2 at a site near its nuclear localization signal inhibits lamin import into the nucleus.

In *Drosophila* three lamin cDNAs, coding for two different proteins, were identified (Gruenbaum et al., 1988; Bossie and Sanders, 1993). The protein encoded by one of these cDNAs (lamin C) is developmentally regulated (D. Riemer et al., unpublished). The other two code for the major *Drosophila* embryo lamin, Dm_0. Lamin* Dm_0 has an apparent molecular mass of 76 kDa as determined by SDS-polyacrylamide gel electrophoresis (PAGE) and is rapidly processed in the cytoplasm into a form migrating at 74 kDa (lamin Dm_1). Lamin Dm_1 is imported into the nucleus where about 50% is post-translationally modified resulting in a slower migrating form (76 kDa) called lamin Dm_2. In vivo pulse-chase studies

*Unless indicated otherwise, for *Drosophila*, the term lamin refers solely to protein products encoded by the *Drosophila* lamin Dm_0 gene.

indicate that lamins Dm_1 and Dm_2 are in equilibrium. Treatment of lamins Dm_1 and Dm_2 with phosphatase results in a single form that comigrates with lamin Dm_1 after SDS-PAGE. In conjunction with results of in vivo labeling, this suggests that lamin Dm_2 arises by specific phosphorylation of lamin Dm_1 (Smith et al., 1987; Smith and Fisher, 1989).

Here, we describe the isolation and characterization of a monoclonal antibody, ADL84, which is specific for *Drosophila* lamin Dm_1 and does not bind lamin Dm_2. We used this antibody to show that isolated interphase lamins occur as mixed dimers, indicating that lamins Dm_1 and Dm_2 interact randomly with each other. Mapping of the epitope for ADL84 shows that the lamin Dm_2-specific phosphorylation takes place in the NH_2-terminal 'head'-domain of the lamin, most likely at serine 25.

MATERIALS AND METHODS

Generation of monoclonal antibodies

We employed an immunization protocol that was shown to enhance the recovery of antibodies against relatively less antigenic epitopes (Matthew and Sandrock, 1987; Vermeersch et al., 1992). At day 1 Balb/c mice were injected intraperitoneally with 150 μg of *Drosophila* lamin Dm_0 produced in *Escherichia coli* (see below) mixed with an equal volume of MPL plus TDM emulsion adjuvant (RIB Immunochemical Research Inc., Hamilton, MT). After 10 minutes, 24 hours and 48 hours, mice were injected with a 2 mg/ml solution of cyclophosphamide (Sigma, St Louis, MO) in 0.9% (w/v) NaCl to a concentration of 100 mg/kg body weight. This set of four injections (one of antigen followed by three of cyclophosphamide) was repeated at day 16. At days 32, 49 and 63, mice were immunized with 150 μg each of authentic interphase *Drosophila* lamins Dm_1 and Dm_2, mixed with adjuvant. At day 66, one mouse was killed, and spleen cells were used for monoclonal antibody (mAb) generation according to standard protocols (Harlow and Lane, 1988).

Hybridomas were tested for specific antibody production using an enzyme linked immunosorbent assay (Harlow and Lane, 1988) with *E. coli* expressed or authentic lamin as antigen. Several anti-*Drosophila* lamin antibody secreting hybridomas were selected and cloned by repeated limiting dilution until greater than 90% of the resulting clones produced the specific antibody. Antibody isotype was determined by enzyme linked immunosorbent assay using isotype-specific secondary antibodies (Fisher Scientific, Pittsburgh, PA).

Expression of the full-length *Drosophila* lamin and deletion mutants in *E. coli*

Full-length *Drosophila* lamin and lamin Dm_0 deletion mutants were expressed in *E. coli* and purified as described elsewhere (N. Stuurman et al., unpublished).

Protein purification

Authentic interphase lamin was purified from 6- to 18-hour-old *Drosophila* embryos essentially as previously described (Lin and Fisher, 1990). All steps were performed at 4°C except when indicated. Frozen, dechorionated embryos were thawed in 9 volumes (one volume equals the starting volume of embryos) of buffer A (50 mM sodium phosphate, pH 8.0, 15 mM $MgCl_2$, 1.0 M sucrose, 0.5 mM DTT) supplemented with protease inhibitors (1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml chymostatin, 2 μg/ml pepstatin-A). Embryos were broken using a Dounce homogenizer (5 strokes, tight pestle) and the homogenate was filtered through two layers of 120 μm nylon mesh. Nuclei were collected by centrifugation for 15 minutes at 20,000 *g* in a swinging-bucket rotor through a cushion of buffer A supplemented with protease inhibitors. After a single wash in buffer A supplemented with protease inhibitors (by resuspension and centrifugation for 10 minutes at 8,000 *g*), nuclei were resuspended in one volume buffer B (10 mM sodium phosphate, pH 8.0, 5 mM $MgCl_2$, 0.5 mM DTT, protease inhibitors). DNase I and RNase A were added to final concentrations of 10 μg/ml each and the suspension was incubated for 15 minutes at 23°C. Nuclei were recovered by centrifugation for 10 minutes at 5,000 *g*, resuspended in 0.9 volumes of buffer C (10 mM sodium phosphate, pH 8.0, 0.1 mM $MgCl_2$, 250 mM sucrose plus protease inhibitors) and Triton X-100 was added to 2% (w/v) from a 20% (w/v) solution in water. After a 10 minute incubation the suspension was centrifuged for 10 minutes at 2,000 *g*. The pellet was resuspended in 0.5 volumes of buffer D (100 mM Tris-HCl, pH 8.1 (4°C), 0.1 mM $MgCl_2$, 250 mM sucrose, 0.1% (w/v) Triton X-100, protease inhibitors) and NaCl was added to a final concentration of 0.5 M from a 1.0 M stock solution. After centrifugation for 10 minutes at 10,000 *g* the supernatant was used immediately for immunoaffinity purification of lamins.

To prepare an anti-*Drosophila* lamin affinity resin, antiserum was raised in rabbits. Specific IgG was affinity-purified from this serum using as the immobilized affinity ligand, bacterially expressed lamin Dm_0 fragment L1-522 (containing residues 1 to 522 of full length lamin Dm_0) covalently bound to glutaraldehyde-activated glass beads (Boehringer, Mannheim, FRG). Bound IgG was eluted using 100 mM glycine-HCl, pH 2.3, 500 mM NaCl, 0.1% (w/v) Triton X-100, thus selecting for anti-lamin IgG which does not bind lamin at low pH. The antibody was cross-linked to Protein-A agarose (Schleicher and Schuell, Keene, NH) using dimethylpimelimidate as described (Harlow and Lane, 1988).

The 0.5 M NaCl extract of nuclease treated, Triton X-100 treated nuclei was mixed with anti-*Drosophila* lamin-Protein A agarose for 4 hours at 4°C. The resin was then washed with 40 column volumes of 20 mM Tris-HCl, pH 8.1 (4°C), 0.5 M NaCl, 5 mM EDTA, 0.1% (w/v) Triton X-100, and bound lamin was eluted with 50 mM glycine-HCl, pH 2.3, 0.5 M NaCl, 0.1% (w/v) Triton X-100. Eluted material was immediately neutralized by addition of Na_2HPO_4 to 50 mM; lamin-containing fractions were pooled and stored at −80°C.

Epitope mapping by random mutagenesis

For random mutagenesis by polymerase chain reaction (PCR) (Zhou et al., 1991), pET-DmLFL (N. Stuurman et al., unpublished) was linearized by digestion with *Eco*RI and used at a final concentration of 50 ng/ml in a PCR reaction containing 10 mM Tris-HCl, pH 9.0 (25°C), 50 mM KCl, 0.1% (w/v) Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 22 units/ml Taq DNA polymerase (Boehringer, Mannheim, FRG), 10% (v/v) dimethylsulfoxide, 0.5 pM T7 primer, 0.5 pM primer 5'-TCTTGCAGTCGTTGG. The reaction was performed in multiple 100 μl-aliquots with the following temperature profile: 5 minutes at 94°C followed by 30 cycles (1 minute and 20 seconds at 94°C, 1 minute at 45°C, 4 minutes at 72°C) and a final extension for 10 minutes at 72°C. The product was phenol/chloroform extracted, ethanol precipitated, digested with *Nde*I and *Hin*dIII, and gel-purified. This fragment was ligated into pET-22b (Novagen, Madison, WI) which had been digested with *Nde*I and *Hin*dIII and had been dephosphorylated. The ligation mixture was electrotransformed into HMS174(DE3)pLysS (Novagen). Colonies were duplicated onto nitrocellulose membranes and induced to produce recombinant protein by placing the filter on an agar plate containing 1 mM IPTG. After three hours at 37°C, the filter was removed and cells were lysed by freezing (5 minutes at −80°C) and thawing (5 minutes at 37°C) twice. Lysed cells were labeled in situ using ADL84 followed by anti-mouse IgG conjugated to alkaline phosphatase (Kierkegaard and Perry Laboratories Inc., Gaithersburg, MD) and a one-solution phosphatase substrate (Kierkegaard and Perry Laboratories Inc.). The same filter was subsequently labeled with affinity-purified polyclonal anti-*Drosophila* lamin IgG followed by anti-rabbit IgG conjugated to horseradish peroxidase. Detection was with an enhanced chemiluminescence system (ECL from Amersham, Arlington Heights, IL).

Alkaline phosphatase-conjugated anti-mouse IgG does not bind rabbit IgG; horseradish peroxidase-conjugated anti-rabbit IgG does not react with mouse IgG (not shown). Colonies which reacted with the polyclonal anti-lamin antibody but not with ADL84 were selected. Plasmids were isolated from the colonies, transformed into XL-1 Blue and sequenced using ssDNA (released with helper phage) and the Sequenase 2.0 kit (United States Biochemical, Cleveland, OH).

SDS-PAGE and immunoblotting

Proteins were separated by SDS-PAGE according to the method of Laemmli (1970) and transferred to nitrocellulose using the method described by Towbin et al. (1979). Blots were stained with a 0.2% (w/v) solution of Ponceau-S (Sigma) and destained with 12 mM HCl to visualize transferred proteins. Blots were equilibrated in PBS containing 0.5% (v/v) Tween-20 and incubated with primary antibody (affinity-purified polyclonal rabbit anti-*Drosophila* lamin IgG at 50 ng/ml, or culture supernatants from hybridomas secreting anti-*Drosophila* lamin antibodies at a 10- to 100-fold dilution) for 2-16 hours at room temperature. Bound antibodies were detected using goat anti-rabbit or goat anti-mouse IgG conjugated to alkaline phosphatase (Kierkegaard and Perry Laboratories Inc., Gaithersburg, MD) and a one-solution phosphatase substrate (Kierkegaard and Perry Laboratories Inc.).

In situ alkaline phosphatase treatment of immunoblots

Lamins Dm_1 and Dm_2 were separated by SDS-PAGE and blotted onto nitrocellulose strips. These strips were incubated for 1 hour at room temperature with 10 mg/ml BSA in PBS containing 0.5% (w/v) Tween-20. They were washed three times with 50 mM glycine, pH 9.5, 0.1% (w/v) Tween-20 and incubated for 90 minutes at 37°C with 500 U/ml calf intestine alkaline phosphatase (Boehringer, Mannheim, FRG) diluted in 50 mM glycine, pH 9.5, 0.1% (w/v) Tween-20. Controls were incubated similarly but without added enzyme. Immunoblot strips were incubated with antibodies essentially as described above; detection of immunoreactivity was with goat anti-mouse IgG conjugated to horseradish peroxidase and a one-solution peroxidase substrate (Kierkegaard and Perry Laboratories Inc.).

Immunoprecipitation

For immunoprecipitation monoclonal antibodies were bound to Protein-G Sepharose (Pharmacia, Piscataway, NJ) by incubating 150 μl of culture supernatant of antibody secreting hybridomas supplemented with 100 mM Tris-HCl, pH 8.0, with 50 μl Protein-G Sepharose for two hours at room temperature. The beads were washed three times with 10 mM sodium phosphate (pH 8.0), 0.5 M NaCl, 5 mM EDTA, 0.1% (w/v) Triton X-100; 15 μl of beads were incubated for 16 hours at room temperature with 30 μl of affinity-purified authentic *Drosophila* lamin Dm_1 and Dm_2 diluted to 2 μg/ml in the same buffer. The Sepharose beads were washed five times with the same buffer and boiled in SDS sample buffer.

Immunofluorescence

Indirect immunofluorescence microscopy on third instar larval salivary gland squash preparations was performed as described previously (Fisher et al., 1982).

Peptide competition assays

Peptides were tested in an antibody capture assay with antigen competition (Harlow and Lane, 1988). Peptides L18-32 (T^{18}STPRPPSAGPQPPP), L18-32:S^{25}P (which has the same primary sequence as L18-32 but is phosphorylated at serine 8), and a control peptide (VGFPVTPQVPLRPMT) were synthesized and HPLC purified to about 80% purity by Neosystem Laboratoire (Strasbourg, France). Microtiter plates were coated with lamin Dmo (0.7 pmol per well) and blocked with BSA. Affinity-purified ADL84 (3.1 nM) in PBS containing 0.1% Tween-20 was preincubated for 75 minutes at 37°C with peptides at the concentrations indicated in Fig. 7. Subsequently, 100 μl of the antibody/peptide mixture was added to each well and incubated for a further 30 minutes at 37°C. After washing with PBS containing 0.1% Tween-20, bound antibody was detected using horseradish peroxidase-conjugated swine anti-mouse Ig (Dako, Rostrup, Denmark) and *o*-phenylenediamine as a substrate. The reaction product was quantified by measuring the absorbance at 495 nm.

RESULTS

ADL84, a monoclonal antibody specific for lamin Dm_1

To study posttranslational modification of the *Drosophila* nuclear lamin, we generated monoclonal antibodies directed against this protein. When immunoblots containing both interphase isoforms (lamins Dm_1 and Dm_2) were probed with these antibodies, one (ADL84) apparently recognized lamin Dm_1 but not lamin Dm_2 (Fig. 1, lane 1). ADL84 also recognized full-length *Drosophila* lamin expressed in bacteria (not shown). Another monoclonal antibody (ADL67) bound both interphase isoforms (lamins Dm_1 and Dm_2) with apparently similar affinity (Fig.1, lane 3). Five other mAbs reacted with both lamins Dm_1 and Dm_2 (not shown).

Previously, it was shown that lamin Dm_2 arises by specific phosphorylation of lamin Dm_1, most likely on one or more serine residues (Smith et al., 1987). To test whether phosphorylation of lamin Dm_2 inhibits binding of ADL84, we treated lamins Dm_1 and Dm_2, separated by SDS-PAGE and immobilized on immunoblots, with alkaline phosphatase before incubation with mAbs. As shown in Fig. 1, lane 2, phosphatase treatment of lamin Dm_2 fixed on nitrocellulose fully restored its reactivity with ADL84. Apparently, the lamin Dm_2-specific phosphorylation decreases substantially the affinity of ADL84 for the *Drosophila* lamin. This suggests that the lamin Dm_2-specific phosphorylation site colocalizes with the epitope for ADL84. Alternatively, specific phosphorylation of lamin Dm_1 to generate lamin Dm_2 causes a structural change in the epitope for ADL84 thus interfering with binding of this antibody; this change would have to survive SDS-PAGE and immunoblot analysis.

Isolated interphase lamins Dm_1 and Dm_2 exist as a random population of mixed dimers

Lamins, like other intermediate filament proteins, form a two-chain (dimeric) coiled-coil by the parallel alignment of

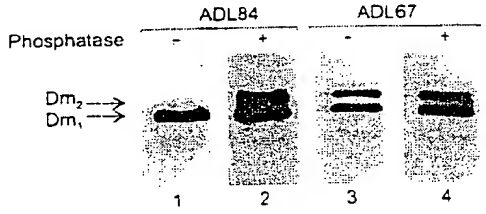


Fig. 1. A monoclonal antibody specific for one of the two *Drosophila* interphase lamin isoforms. Authentic *Drosophila* lamins affinity-purified from 6- to 18-hour-old embryos were separated on an SDS-8% polyacrylamide gel and blotted to nitrocellulose. Separate blot strips were incubated with (lanes 2 and 4) or without (lanes 1 and 3) alkaline phosphatase, followed by ADL84 or ADL67 as indicated. Bound mAbs were detected using a colorimetric assay system. Migration positions of lamin Dm_2 (76 kDa) and lamin Dm_1 (74 kDa) are indicated on the left-hand side of the figure.

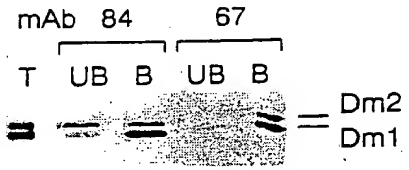


Fig. 2. Immunoprecipitation of isolated authentic *Drosophila* lamins with ADL84. Isolated lamins were immunoprecipitated with the lamin Dm_1-specific mAb ADL84, or with ADL67 which recognizes both lamins Dm_1 and Dm_2. Immunoprecipitation was carried out under conditions that allow lamins to form dimers (see text). Shown is an immunoblot probed with affinity-purified polyclonal anti-*Drosophila* lamin antibodies which recognize both lamins Dm_1 and Dm_2 equally. Lane T, total before immunoprecipitation; lanes UB, unbound material after immunoprecipitation with the indicated mAb; lanes B, material bound after immunoprecipitation with the indicated mAb. Equivalent amounts of total, bound and unbound material were loaded. Migration positions of lamins Dm_1 and Dm_2 are indicated on the right-hand side of the figure.

molecules (Aebi et al., 1986; Parry et al., 1986). This raised the question of whether the two interphase isoforms, lamins Dm_1 and Dm_2, form obligate homodimers, obligate heterodimers, or show no preference for themselves or each other. We addressed this question using ADL84 to immunoprecipitate selectively lamin Dm_1 under conditions where dimer formation takes place but no higher order polymerization is evident (Lin and Fisher, 1990). If the lamin isoforms formed obligate homodimers, only lamin Dm_1 would be found in the fraction bound to ADL84. In the case of obligate heterodimers, lamins Dm_1 and Dm_2 would be found in equimolar amounts in the bound fraction. If the lamin isoforms showed no preference for themselves or each other, one would expect one molecule of lamin Dm_2 for two molecules of lamin Dm_1 (a lamin Dm_2 to Dm_1 ratio of 0.50) in the ADL84 bound fraction.

Affinity-purified interphase lamins were diluted in 10 mM phosphate buffer, pH 8.0 containing 0.5 M NaCl, 5 mM EDTA and 0.1% (w/v) Triton X-100 (a condition which allows dimer formation but prevents higher-order polymerization) and mixed with Protein G-Sepharose beads which had been preadsorbed with either ADL84 or ADL67. ADL67 apparently reacts with both interphase lamin isoforms equally (see Fig. 1). It is evident from Fig. 2 (lane 84, UB) that lamin Dm_1 was efficiently depleted from the unbound fraction by immunoprecipitation with ADL84, while a substantial amount of lamin Dm_2 remained. Comparison with the unbound fraction after immunoprecipitation with ADL67 (which binds both lamin isoforms) demonstrated the specificity of ADL84 for lamin Dm_1 under the conditions used for immunoprecipitation. The fraction bound to ADL84 was enriched in lamin Dm_1 but contained a substantial amount of lamin Dm_2 (Fig. 2, lane 84, B). Densitometry revealed that the ratio of lamins Dm_2:Dm_1 (normalized to the ratio of these isoforms in the starting material) was 0.53 ± 0.07 ($n=4$). The ratio of lamins Dm_2:Dm_1 bound to mAb ADL67 (normalized to

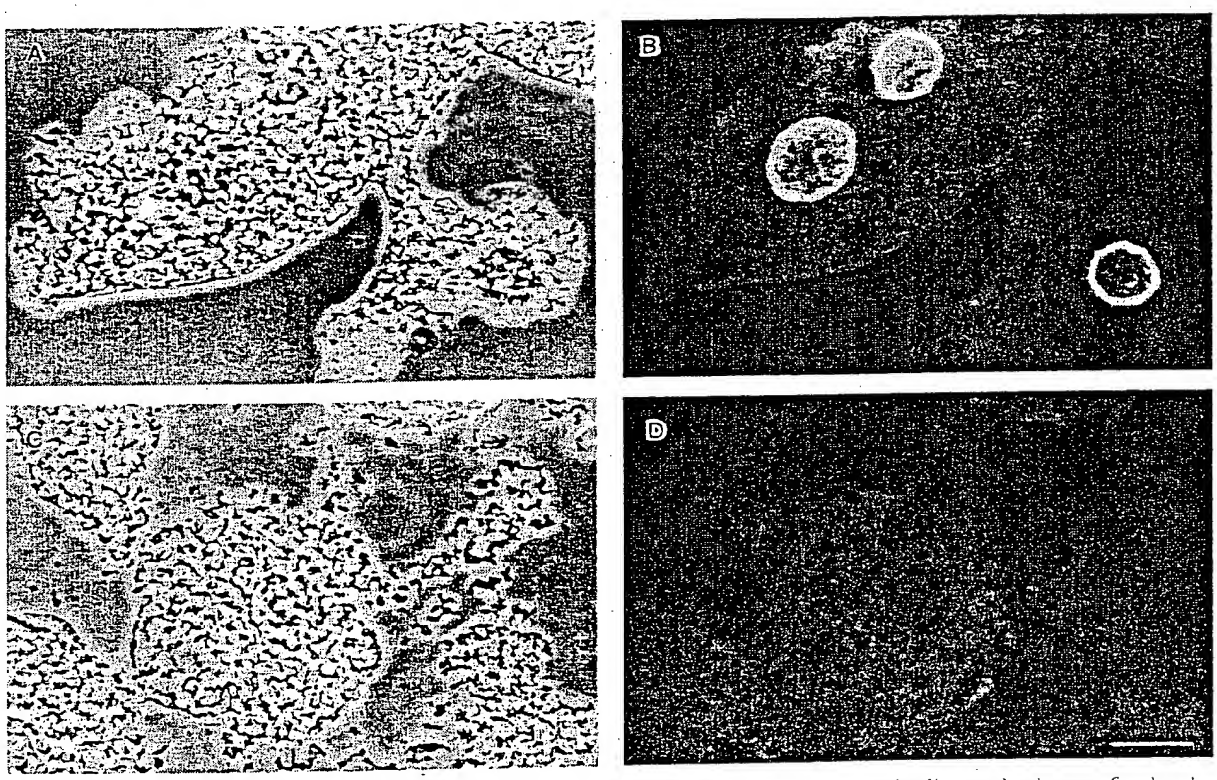


Fig. 3. Indirect immunofluorescence localization of ADL84 reactivity in salivary gland cells. Squashed salivary glands were fixed and incubated with culture supernatant from ADL84 (A and B) or culture supernatant from a hybridoma secreting an unrelated Ig (C and D). Bound antibody was detected with rhodamine conjugated anti-mouse IgG. Phase contrast (A and C) and fluorescence micrographs (B and D) are shown. Bar in D, 20 μm (applies to all panels).

the ratio of the two forms in the starting material) was 0.96±0.03 (n=4). Evidently, isolated lamins Dm_1 and Dm_2 do not show any preference for themselves or each other at the level of dimer formation. Moreover, ADL84 and ADL67 react with nondenatured lamins in solution as well as with SDS-denatured lamins on immunoblots.

Indirect immunofluorescence analysis of *Drosophila* cells and tissues with ADL84

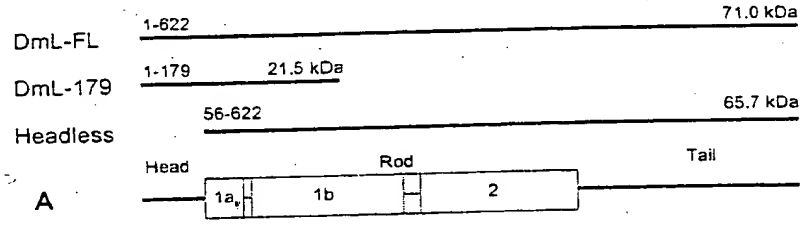
Tissue culture supernatant of ADL84 was used for in situ localization. Results (Fig. 3) show that ADL84 binds to the nuclear periphery of *Drosophila* salivary gland nuclei, suggesting that the antibody also recognizes lamin Dm_1 in situ. Previously, it was shown that most, if not all, cell nuclei of *Drosophila melanogaster* bind a polyclonal antibody specific for lamins Dm_1 and Dm_2 (Whalen et al., 1991). To determine if any cells or tissues lacked lamin Dm_1, ADL84 was used to label cryosections through all stages of the *Drosophila* life cycle. All detectable nuclei were labeled with ADL84 (not shown). To exclude in vitro phosphatase activity, stained cryosections of embryos were analyzed by immunoblotting. No alteration in the ratio of lamins Dm_1 and Dm_2 was seen (not shown) indicating that the observed staining pattern was not caused by an in vitro conversion of lamin Dm_2 into Dm_1. These data suggest that all individual nuclei contain a significant amount of lamin Dm_1.

Mapping the ADL84 epitope by random mutagenesis

To gain insight into the location of the lamin Dm_2-specific phosphorylation site, we mapped the epitope for ADL84. First, we tested binding of ADL84 to two deletion mutants of the *Drosophila* lamin expressed in *E. coli* (schematically represented in Fig. 4A). ADL84 recognized a fragment formed by the 179 NH_2-terminal amino acids of *Drosophila* lamin Dm_0 (Fig. 4B, lanes 3). ADL84 did not bind to the fragment designated Headless which consists of all but the first 56 NH_2-terminal lamin amino acids (Fig. 4B, lanes 2). Evidently, the epitope for ADL84 is located in the first 179 amino acids of the molecule, and is most likely within the NH_2-terminal 56 amino acids.

To map the ADL84 epitope more precisely, random mutations were PCR-generated in the coding region for DmL-179. The mutated fragments were ligated into pET-22b and transformed into a host which expressed the T7 lysozyme gene (HMS174(DE3)pLysS). We chose a host containing the T7 lysozyme gene to ensure cell lysis by simple freezing and thawing. Colonies were transferred to nitrocellulose filters and expression of the mutant protein was induced by transfer of the filters to plates containing IPTG. After induction, cells were lysed and colonies were selected which could be labeled in situ with affinity-purified rabbit anti-lamin antibodies (Fig. 5, lower panel) but not with ADL84 (Fig. 5, upper panel). Of about 2,000 colonies analyzed, 12 satisfied these criteria. Cells from all 12 colonies were grown separately in suspension culture, induced to express the mutant protein with IPTG and total cell lysates were analyzed by immunoblotting using ADL84 (Fig. 6 upper panel) and affinity-purified polyclonal anti-lamin antibody (Fig. 6, lower panel). All showed a highly reduced reactivity of the mutant protein towards ADL84 but maintained full reactivity with the polyclonal anti-lamin antibody. Two representative examples are shown (Fig. 6, lanes 1 and 2).

Sequencing of these 12 clones revealed that all were mutated in an area coding for amino acids 22-28. All mutations resulted in an amino acid change (Table 1). Three mutants were sequenced throughout their coding sequence and were found not to have any additional mutations. These data indicate that



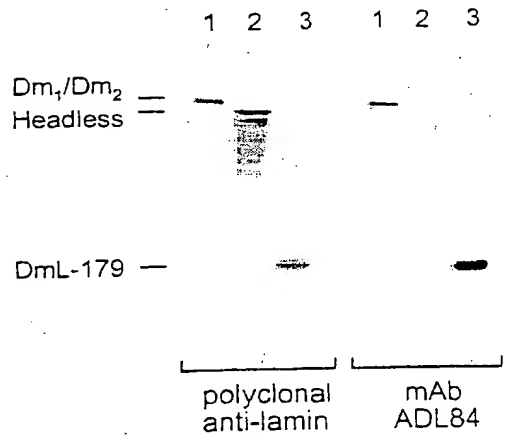


Fig. 4. Binding of ADL84 to full-length *Drosophila* lamin and deletion mutants expressed in *E. coli*. (A) Schematic representation of the proteins used. Numbers at the left-hand side indicate the amino acids of the full-length protein contained in each construct. On the right-hand side the molecular mass of the protein product of each construct is indicated in kDa. The lowest diagram in the panel schematically represents the structure of the lamin molecule. (B) Immunoblots probed with anti-lamin antibodies. Identical blots were probed with affinity-purified polyclonal anti-*Drosophila* lamin IgG or with ADL84, as indicated. Lanes 1, affinity-purified authentic lamins Dm_1 and Dm_2. Lanes 2, product of the Headless mutant. Lanes 3, product of the DmL-179 construct. Approximately the same amount of protein was loaded in each lane. Extra bands in lane 2 are likely to be breakdown products of the Headless mutant, generated within bacteria during production of the protein. Migration positions of authentic lamins (Dm_1 and Dm_2), Headless and DmL-179 are indicated on the left-hand side of the figure.

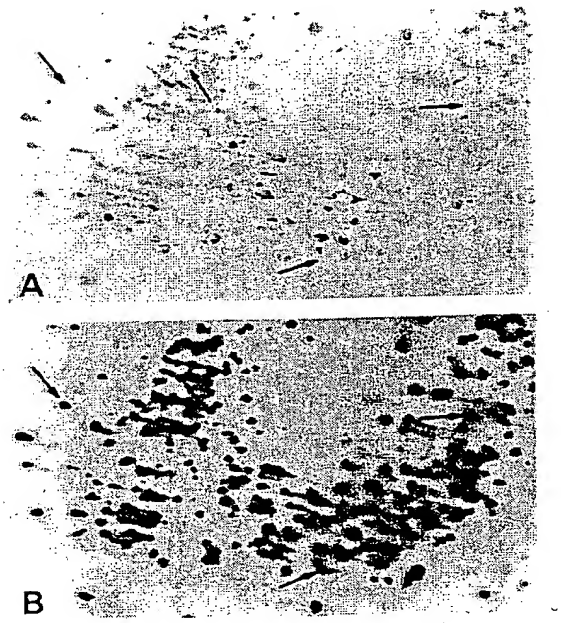


Fig. 5. Epitope mapping of ADL84; screening for point mutants which abolish binding to ADL84. Random mutations were generated in the coding sequence of DmL-179. The plasmid was transformed into *E. coli* strain HMS174(DE3)pLysS. Resulting colonies were transferred to nitrocellulose, protein expression was induced with IPTG and colonies were lysed by freezing and thawing. Filters were double-labeled with ADL84 (using alkaline phosphatase-conjugated anti-mouse IgG secondary antibodies and colorimetric detection; A) and polyclonal affinity-purified rabbit anti-lamin IgG (using peroxidase-conjugated anti-rabbit secondary antibodies and enhanced chemiluminescence; B). Arrows indicate colonies producing mutated DmL-179 that do not bind ADL84.

the epitope for ADL84 is formed by amino acid sequence R^{22}PPSAGP. The location of a serine in the middle of this sequence suggests that this serine is the site specifically phosphorylated in lamin Dm_2.

Peptide competition experiments demonstrate that phosphorylation of serine 25 inhibits binding to ADL84

To corroborate conclusions drawn from epitope mapping by random mutagenesis, we synthesized the peptide representing amino acids 18-32 of lamin Dm_0 (L18-32) and tested its ability to bind to ADL84 in a competition assay. As shown in Fig. 7, this peptide inhibits binding of ADL84 to lamin Dm_0 immobilized on microtiter plates; 50% inhibition is observed at about a 13-fold molar excess of peptide over antibody. An unrelated control peptide did not inhibit binding of ADL84 to lamin Dm_0 (Fig. 7). Similarly, L18-32 did not inhibit binding of another anti-*Drosophila* lamin mAb to lamin Dm_0 (not shown).

To determine whether phosphorylation of serine 25 influenced binding of ADL84 we also tested a variant of the peptide L18-32 that was chemically phosphorylated at the serine residue corresponding to serine 25 in the full length lamin. This phosphorylated peptide inhibited binding of ADL84 to immobilized lamin Dm_0 with an efficiency that was about 19-fold lower than the unphosphorylated peptide (Fig. 7); 50% inhibition occurred at a 250-fold molar excess of the phosphorylated peptide over antibody. Alkaline phosphatase treatment of the chemically phosphorylated peptide increased its ability to compete for binding to ADL84 to levels comparable to the original, unphosphorylated peptide (50% inhibition at an 18-fold molar excess of peptide over antibody). Thus, we conclude that the epitope for ADL84 is contained within amino acids 18-32 of lamin Dm_0 and that phosphorylation of serine 25 inhibits lamin binding to ADL84.

Fig. 6. Epitope mapping of ADL84; analysis of selected mutants by immunoblotting. Selected colonies (as shown in Fig. 5) were grown in liquid culture, induced with IPTG, lysed by boiling in SDS-containing sample loading buffer and separated on SDS-10% polyacrylamide gels. Duplicate gels were transferred to nitrocellulose and probed with ADL84 (A) or with polyclonal affinity-purified rabbit anti-*Drosophila* lamin IgG (B). Lanes 1,2, selected mutants; lane WT, colony containing the unmodified plasmid coding for DmL-179.

DISCUSSION

Interphase *Drosophila* lamins Dm_1 and Dm_2 are highly phosphorylated (2-3 phosphates per molecule). The function of this phosphorylation is unknown. Using a procedure designed to

Table 1. Mutations that disrupt binding of ADL84 to *Drosophila* lamin*

Frequency	Mutation in the DNA	Mutation in the Protein
3	G^{194} A	R^{22} W
1	C^{195} A	R^{22} L
3	A^{203} G	S^{25} P
1	G^{191} T	P^{21} T
	A^{203} G	S^{25} P
1	A^{203} G	S^{25} P
	G^{249} A	A^{40} V
1	C^{204} A	S^{25} L
1	G^{213} A	P^{28} L
1	G^{213} A	P^{28} L
	T^{567} A	K^{146} M

*Mutants with reduced binding to ADL84 as determined by immunoblotting were sequenced throughout the region coding for the head domain (lamin amino acids 1-56). Three mutants (R^{22} W, S^{25} P, and P^{28} L) were sequenced throughout their coding region and found not to contain any additional mutations.

Amino acid sequence of *Drosophila* lamin Dm_0 head; the probable ADL84 epitope is shown in bold: M^1SSKSRRAGT ATPQPGNTST PR^{22}PPSAGPQP PPPSTHSQTA SSPLSPTRHS RVAEKV^{56}.

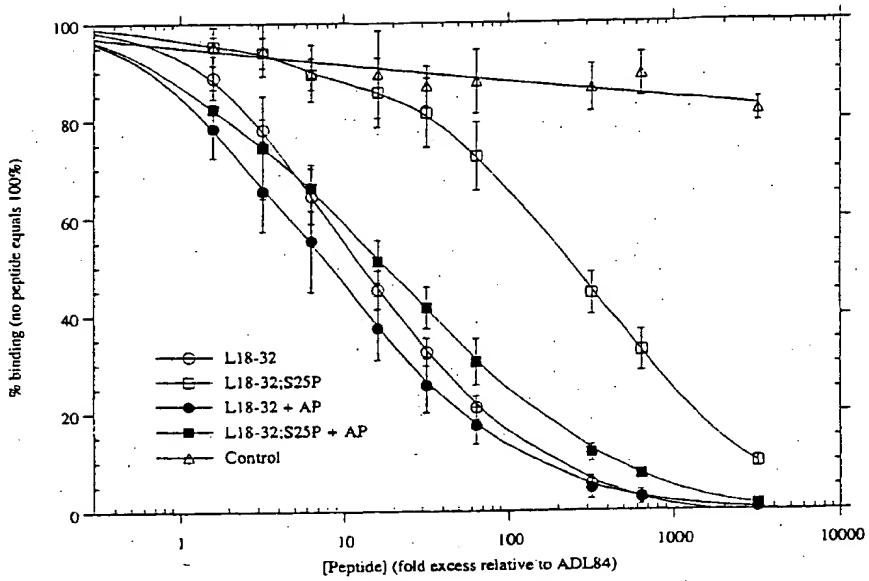


Fig. 7. Phosphorylation of serine 25 decreases the ability of peptide L18-32 to inhibit binding of ADL84 to lamin Dm_0. Purified ADL84 was preincubated with peptide L18-32, a peptide with the same primary sequence but chemically phosphorylated at serine 25 of the full length lamin sequence (L18-32;S25P), either peptide previously incubated with calf intestinal alkaline phosphatase (L18-32 + AP and L18-32;S25P + AP) or a control peptide. Subsequently the antibody/peptide mixtures were incubated with bacterially expressed lamin Dm_0 immobilized on microtiter plates. The amount of ADL84 bound to the microtiter plate was determined using horseradish peroxidase-conjugated secondary antibodies. Signals without added peptide were set at 100%. Peptide L18-32 inhibited binding of ADL84 to lamin Dm_0, whereas peptide L18-32;S25P was much less effective. Alkaline phosphatase treatment of L18-32;S25P largely restored its ability to inhibit binding of ADL84 to lamin Dm_0.

generate mAbs specific for relatively less antigenic sites, we obtained an antibody, ADL84, that reacts with an epitope involved in the posttranslational conversion (via phosphorylation) of lamin Dm_1 to lamin Dm_2. When this site is modified, binding of ADL84 to lamin is largely or completely eliminated. The antibody is specific for both the denatured *Drosophila* lamin Dm_1 presented on immunoblots after SDS-PAGE (Fig. 1), and nondenatured lamin Dm_1 in solution (Fig. 2). Moreover, ADL84 recognizes lamin Dm_2 immobilized on nitrocellulose blots after enzymatic dephosphorylation (Fig. 1) and binds to the nuclear periphery when used for in situ labeling (Fig. 3).

Lamins Dm_1 and Dm_2 have been observed after immunoblot analysis of all *Drosophila* cells and tissues analyzed to date. This includes embryos (Smith et al., 1987), Schneider 2 and Kc tissue culture cells (Smith et al., 1987; L. Lin-Mantell and P. A. Fisher, unpublished) and various larval, pupal and adult tissues (Whalen et al., 1991) including ovaries (Smith and Fisher, 1989). Results of in situ localization studies (Fig. 3) showing uniform distribution of lamin Dm_1 throughout all nuclei examined support the notion that lamins Dm_1 and Dm_2 are present in all interphase cells. This ubiquitous distribution of both lamin isoforms during development and its precise regulation suggest that lamin Dm_2-specific phosphorylation has a general role in lamin function. Based on immunoprecipitation with ADL84, we can now conclude that lamins Dm_1 and Dm_2 interact to form heterodimers in vitro. It seems highly likely that such interactions also take place within the nuclear envelope in vivo.

We used ADL84 to determine the likely phosphorylation site involved in conversion of lamin Dm_1 to lamin Dm_2. Based on phosphoamino acid analysis, it was apparent that this conversion was mediated by serine phosphorylation (Smith et al., 1987). Random mutagenesis followed by screening for selective loss of reactivity with ADL84 (Fig. 5) suggested that the epitope for ADL84 consists of the sequence R^{22}PPSAGP (Fig. 6 and Table 1). We confirmed this by showing that a peptide consisting of amino acids 18-32 of the lamin Dm_0 sequence inhibits binding of ADL84 to lamin Dm_0. In the middle of the epitope is a single serine residue, located at position 25. Phosphorylation of this serine reduced the ability of the peptide to inhibit binding of ADL84 to lamin Dm_0 by about 19-fold. The residual inhibition might be caused by incomplete chemical phosphorylation of the peptide, contaminating phosphatase activity during the assay, or it might reflect a reduced, but measurable affinity of the phosphorylated peptide for ADL84. These data indicate that phosphorylation of serine 25 distinguishes lamin Dm_2 from lamin Dm_1.

The methodology used to define the ADL84-epitope (random mutagenesis followed by screening with both monoclonal and polyclonal antibodies) was both rapid and efficient. A similar approach was used by Ikeda et al. (1992) to map epitopes of two anti-*E. coli* recA protein mAbs. We modified their method in a number of ways. By substituting a T7 RNA polymerase-dependent expression system for λgt11, we were able to both express and sequence mutated proteins without the need for subcloning. Use of an *E. coli* strain expressing the gene for bacteriophage T7 lysozyme facilitated antibody screening in that colonies could be efficiently lysed simply by freezing and thawing. The utility of this approach for antibody screening of expressed protein fragments was demonstrated in this article. This approach may also prove useful for high-resolution mapping of polypeptide domains involved in specific protein-protein interactions.

In conclusion, the most immediate question regarding the conversion of lamin Dm_1 to lamin Dm_2 concerns its physiological/functional significance. The highly dynamic nature of the event as well as the apparently quantitative conversion of lamin Dm_2 to lamin Dm_1 during heat shock (Smith et al., 1987) suggests that it may be involved in such processes as growth of the nuclear envelope during interphase and/or regulation of gene expression. The identification of the site involved in the conversion of lamin Dm_1 to lamin Dm_2 will permit us to address this question directly using genetic analyses of both tissue culture cells and whole organisms.

The authors thank Mike Frohman (Stony Brook, NY) for the stimulating discussions concerning the use of PCR-mediated random mutagenesis, Sergei Bogachev (Novosibirsk, Russia) for performing

the in situ labeling experiments as well as Ueli Aebi (Basel, Switzerland) and Klaus Weber (Goettingen, FRG) for support and discussion, and Toni Daraio (Stony Brook, NY) for help in preparing the manuscript. These studies were supported by a research grant from the National Institutes of Health (USA). N.S. was supported in part by a Long Term Fellowship from the Human Frontier Science Program Organization and in part by a Fulbright Scholarship.

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(Received 11 April 1995 - Accepted 14 June 1995)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Ni et al.	Docket No.: PF210D1
Application No.: 09/227,854	Group Art Unit: 1646
Filed: January 11, 1999	Examiner: S. Prasad
For: HUMAN CHEMOTACTIC CYTOKINE I POLYPEPTIDES (AS AMENDED)	

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

The paragraph beginning on page 7, line 4, has been amended as follows:

Figure 3 ~~shows structural and functional features of the polypeptide of the present invention deduced by the indicated techniques, as a function of amino acid sequence.~~ depicts the purification of the protein of the invention on a gel.

In the Claims:

Claims 35-41, 44 and 60-68 have been amended as follows:

35. (Once Amended) An isolated polypeptide ~~comprising~~ consisting of at least 30 contiguous amino acid residues of SEQ ID NO:2.

36. (Once Amended) The ~~An~~ isolated polypeptide of claim 35, wherein said polypeptide consists of ~~comprising~~ at least 50 contiguous amino acid residues of SEQ ID NO:2.

37. (Once Amended) An isolated polypeptide ~~of claim 36~~ comprising amino acids 2 to 92 ~~in~~ of SEQ ID NO:2.

38. (Once Amended) The ~~An~~ isolated polypeptide of claim 37, wherein said polypeptide comprises ~~comprising~~ amino acids 1 to 92 ~~in~~ of SEQ ID NO:2.

39. (Once Amended) ~~An~~ The isolated polypeptide of claim 35 ~~further comprising~~ fused to a heterologous polypeptide ~~sequence~~.

40. (Once Amended) An isolated polypeptide ~~comprising~~ consisting of at least 30 contiguous amino acid residues of the polypeptide encoded by the human cDNA contained in ATCC Deposit No. 97304.

41. (Once Amended) The isolated polypeptide of claim 40, wherein said polypeptide consists of ~~comprising~~ at least 50 contiguous amino acid residues of the polypeptide encoded by the human cDNA contained in ATCC Deposit No. 97304.

44. (Once Amended) The isolated polypeptide of claim 40 ~~further comprising~~ fused to a heterologous polypeptide ~~sequence~~.

60. (Once Amended) An isolated first polypeptide ~~comprising a first amino acid sequence~~ 90% or more identical to a second ~~amino acid sequence~~ polypeptide selected from the group consisting of:

(a) amino acid residues 2 to 92 of SEQ ID NO:2;

(b) amino acid residues 1 to 92 of SEQ ID NO:2;

(c) the amino acid sequence of the mature polypeptide encoded by the cDNA in ATCC Deposit No. 97304; and

(d) the amino acid sequence of the full length polypeptide encoded by the cDNA in ATCC Deposit No. 97304.

61. (Once Amended) The isolated first polypeptide of claim 60 wherein the ~~first amino acid sequence is 90% or more identical to the~~ second ~~amino acid sequence~~ polypeptide is (a).

62. (Once Amended) The isolated first polypeptide of claim 60 wherein the ~~first amino acid sequence is 90% or more identical to the~~ second ~~amino acid sequence~~ polypeptide is (b).

63. (Once Amended) The isolated first polypeptide of claim 60 wherein the ~~first amino acid sequence is 90% or more identical to the~~ second ~~amino acid sequence~~ polypeptide is (c).

64. (Once Amended) The isolated first polypeptide of claim 60 wherein the ~~first amino acid sequence is 90% or more identical to the~~ second ~~amino acid sequence~~ polypeptide is (d).

65. (Once Amended) The isolated first polypeptide of claim 60 wherein ~~the~~ said first ~~amino acid sequence~~ polypeptide is 95% or more identical to ~~the second amino acid sequence~~ (a).

66. (Once Amended) The isolated first polypeptide of claim 60 wherein ~~the~~ said first ~~amino acid sequence~~ polypeptide is 95% or more identical to ~~the second amino acid sequence~~ (b).

67. (Once Amended) The isolated first polypeptide of claim 60 wherein ~~the~~ said first ~~amino acid sequence~~ polypeptide is 95% or more identical to ~~the second amino acid sequence~~ (c).

68. (Once Amended) The isolated first polypeptide of claim 60 wherein ~~the~~ said first ~~amino acid sequence~~ polypeptide is 95% or more identical to ~~the second amino acid sequence~~ (d).

69. (Once Amended) The isolated first polypeptide of claim 60 ~~further comprising~~ fused to a heterologous polypeptide ~~sequence~~.